Purification and Characterization of Active Human Cytomegalovirus Alkaline Nuclease pUL98 and its Use in Developing a High Throughput Assay to Test Potential Inhibitors

Undergraduate Research Thesis

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Abstract

Human cytomegalovirus (CMV) is a widespread human herpesvirus responsible for a number of complications and diseases, particularly in immunocompromised individuals such as AIDS patients, organ transplant recipients, and patients undergoing treatment for cancer. Although there are antiviral compounds that have been approved for the treatment of CMV, there are a number of issues with these current drugs including limited bioavailability, significant toxicity, and high rates of resistance. Additionally, all of these drugs have the same target, the viral DNA polymerase. Thus, there is a need for new antivirals that have novel targets and lower toxicity. The herpesvirus alkaline nuclease, pUL98 in CMV, represents a potential novel target for the development of new antivirals. This protein is critical for efficient replication of the virus and has no closely related human homolog. The goal of this research was to purify and characterize active pUL98, which was then used to design a miniaturized, high throughput assay for screening potential inhibitors of the nuclease. A fusion construct consisting of a double histidine tag incorporated into the N-terminus of pUL98 facilitated purification of the protein by sequential passage over Ni-chelating and Mono Q anion exchange columns. Activity of the nuclease was confirmed using an assay measuring the ability to release soluble, radioactive nucleotides from uniformly labelled DNA. Several compounds have been identified via *in silico* modeling that are predicted to interact with and thus interfere with the active site of pUL98. Two of these compounds, Acid Blue 129 and Acid Blue 40, have been found to inhibit CMV replication. Using the newly designed assay, it was determined that Acid Blue 129 has a greater inhibitory effect on the nuclease activity of pUL98 (IC_{50}= 9.3 μM) than does Acid Blue 40 (IC_{50}- = 149 μM). Both the new assay and *in silico* modeling to identify potential inhibitors of a protein were validated in the course of these experiments. Furthermore, inhibition of pUL98 may be the

mechanism by which Acid Blue 129, and the larger class of anthraquinone derivatives, has anti-CMV activity. Further studies are needed to identify what genetic changes result in resistance to Acid Blue 129, thus elucidating the mechanism of inhibition.

Acknowledgements

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Introduction

Herpesviruses: Impact on Health

Viruses are entities that identify, target, infect, and subvert the machinery of host cells in order to replicate and form new, infectious particles. A number of complications arise from viral infection of cells--directly as a consequence of host cell destruction and from the downstream effects of host infection and viral reactivation. Some of the more common viruses that impact human health are those of the order *Herpesvirales*, specifically the family *Herpesviridae* (Figure 1). Those herpesviruses that infect humans are members of this family, and they are responsible for a number of diseases, issues, and complications in human health. There are eight human herpesvirus species comprising nine viruses, which include herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), Epstein-Barr virus (EBV), varicella zoster virus (VZV), human cytomegalovirus (CMV), human herpesvirus 6A, 6B, and 7, and Kaposi sarcoma-associated herpesvirus (KSHV), otherwise known as human herpesvirus 8 (HHV8). The specific effects of infection by a given virus depend on many parameters, including the site of infection, the age and immune competence of the infected individual, and whether the infection is due to a primary infection or one that results from reactivation of latent virus in that host.

Herpes simplex virus types 1 and 2 (HSV-1/2) can have clinical presentations that vary greatly depending on these different factors—ranging from gingivostomatitis (cold sores), genital blisters (herpes labialis), and lesions on the fingers (whitlow), to life-threatening encephalitis, meningitis, or systemic herpes infection in neonates (9). Chickenpox, a common childhood illness, is the result of a primary VZV infection. Reactivation of the virus in adults infected during childhood causes shingles, a painful disease that can leave lasting effects and neurological pain in patients (39). EBV is commonly associated with infectious mononucleosis

in adolescents and adults (15), but also is linked to a variety of cancers such as Burkitt lymphoma, CNS lymphoma, and nasopharyngeal carcinoma (15, 16). HHV8 is thought to be at least partially responsible for Kaposi's sarcoma and other proliferative diseases in immonsuppressed men (35). As for many viral infections, the severity of these clinical manifestations is largely affected by the immune status of the host. Those individuals that are immunocompromised, whether from disease, medication, aging, or other causes, generally experience significantly more severe disease states (9, 35, 43).

Compounding the variety of different diseases and the associated viral-induced effects on human hosts is the prevalence of the different herpesviruses. HSV-1 and HSV-2 are quite common, and are estimated to infect between 65-90% of the world's adult population (9). In the United States, the incidence of HSV-1 increases as an individual ages, from 26.3% at 6 years to 90% in those 70 years or older (9). For HSV-2, the incidence ranges from negligible to 20-40% at 40 years (9). The incidence of EBV is similar to that of HSV-1 with >90% infected by adulthood throughout the world (15). Of note is the fact that the exact prevalence of the different viruses varies by geographic region. KSHV is estimated to have a prevalence of 1-3% in North American blood donors but a prevalence greater than 70% in regions of Africa and in the Mediterranean where it is considered endemic (35). Thus, in both developed and developing countries, human herpesviruses have significant roles in the well-being of human populations.

It should be noted that herpesviruses infect animal hosts beyond humans. There are over 300 identified herpesviruses, the majority of which have only been discovered using PCR amplification (11). These herpesviruses infect a broad range of other organisms including rats, gorillas, sheep, cattle, primates, pigs, dogs, horses, fish, birds, and turtles (5, 12). The host range extends over most of the vertebrate lineage and even includes two herpesviruses found in

invertebrates (11). Even the non-human herpesviruses have an indirect effect on humans, as a number of the host organisms constitute livestock species of significant use and value.

Classification of Herpesviruses

Despite significant variations in host range, tropism, and clinical manifestation, there are unifying similarities between the different herpesviruses, the extent of which is determined by the taxonomic relationships between the different viruses. The structure of the herpesvirus infectious particle (virion) is conserved across all herpesviruses, and is the unifying characteristic defining *Herpesvirales* (11). Genetically, however, only two genes are homologous at the amino acid level across all members of *Herpesvirales*: DNA polymerase and the ATPase subunit of terminase (11). Among viruses, neither of these genes is unique to herpesviruses, though the ATPase subunit of terminase is the closest to being unique, as it is conserved only in T4-like bacteriophages in addition to herpesviruses (12). Analysis of the family *Herpesviridae* shows that a set of roughly 44 genes has been passed down from the common ancestor to the human herpes viruses, though some of these core genes have been lost over time in different viruses (11). Genes that are not part of this core generally represent gene products that allow the virus to occupy its specific niche (11).

Based on recommendations of International Committee on Taxonomy of Viruses (ICTV), *Herpesviridae* can be further divided into three subfamilies that includes *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae* (Figure 1) (12). HSV-1, HSV-2 and VZV are members of *Alphaherpesvirinae* (3). Human cytomegalovirus and human herpesviruses 6A, 6B, and 7 are members of the *Betaherpesvirinae* (3). The *Gammaherpesvirinae* subfamily contains EBV and KSHV (3). The ICTV constantly reevaluates the delineations and increasingly relies on DNA sequence comparisons to determine evolutionary relationships (10).

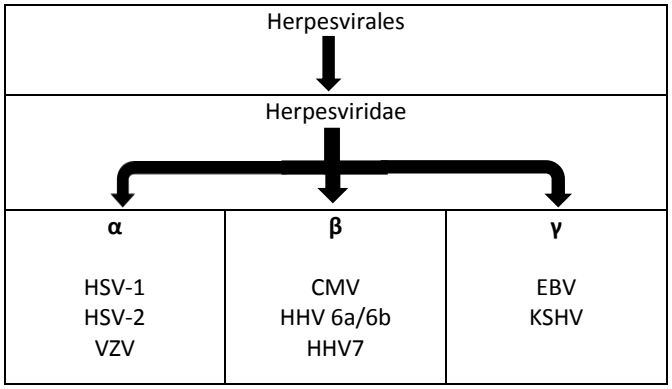

Figure 1 The phylogeny of the human herpes viruses. Within the order *Herpesvirales*, all human herpes viruses belong to the family *Herpesviridae*, which can be further subdivided into three separate subfamilies, α, β, and γ.

General Structure and Replication of Herpesviruses

The *Herpesviridae* family of viruses are united not only by having a specific physical particle structure that is readily discernable by electron microscopy, but also by alternating between two kinds of life-cycles—1) productive replication, characterized by expression of most of the viral genes and usually resulting in the lysis of the infected cells, and 2) latency, characterized by a more restricted pattern of gene expression that fails to result in the production of infectious virions and which usually spares the cell from death (Figure 2). Additionally, many of the genomes of the herpesviruses share their overall architecture and are comprised of two different unique sequences, known as unique long (UL) and unique short (US), that are flanked by inverted repeats at both ends of each of the unique sequences (11, 40, 45). The region of the genome where a gene locus resides and its relative order within that region determines both the name of the gene and its protein (i.e. UL98 for the gene and pUL98 for the protein).

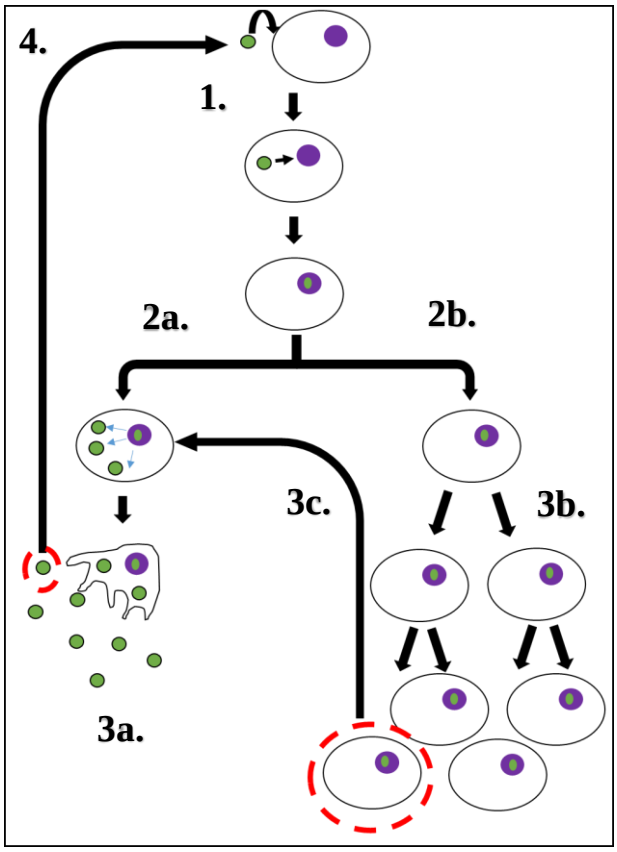

Figure 2 Overview of the lytic and latent phases of the viral replication cycle. 1) The virus comes into contact with and specifically recognizes a host cell, enters the cell, and the nucleocapsid transfers its DNA to the nucleus. 2a) If the virus enters into the lytic pathway, it expresses most of the genes it encodes and replicates the genome. Viral DNA packaging and virion maturation then occur. 3a) Production of the virus continues until the host cell lyses. 2b) If the virus enters into the latent pathway, the viral genome becomes associated with histones and remains largely quiescent. Only a limited set of viral genes are expressed and translated. Depending on the virus, the infected cells (such as neurons) do not divide and the viral genome remains. 3b) For other types of herpesviruses, the infected cell divides, but the viral genome replicates with cellular chromosomes, links to the chromosomes, and remains in cells following division. 3c) At times, the virus can be reactivated due to specific conditions in the cell or its environment. The virus then enters into the lytic pathway. 4) The viral progeny are released, and the process begins again.

Structure of Virions

Herpesviruses share a specific set of core features that make up the structure of the virion. These include a double-stranded, linear DNA genome, the highly structured protein capsid surrounding that nucleic acid, the tegument, which are proteins that lie outside the capsid but within the outermost layer, and a lipid-containing envelope (Figure 3). Compared to many other viruses, the size of the genome in these viruses is relatively large (3). In addition to large genomes, herpes virions are also large in physical size — for example, virions of HSV-1 are

roughly 125 nm in diameter with a mass of about 200 MDa (8). The general characteristics of the capsid are conserved across the herpesviruses, at least in the *Herpesviridae* family (26). Each capsid has an icosahedral symmetry and is comprised of a number of proteins assembled into repeating units that compose the pentomers and hexomers (26). Unless specifically noted, all proteins in this section are referred to by their HSV-1 designation. Of the 12 pentomers at the vertices of the icosahedron, eleven of them are identical and are formed by the pUL19 subunit. The twelfth vertex is formed from a unique dodecameric portal complex containing the UL6 protein, through which the DNA is inserted (8). pUL19 also serves as the main component of the 150 hexomeric capsomers (the functional subunit of a capsid), with other proteins (e.g. pUL18 and pUL38) linking the capsomers together (26).

The next layer of structure is comprised of a relatively large number proteins, and is known as the tegument (Figure 3b). This structure resides between the capsid and the membrane, and serves both regulatory and structural functions, including linking the capsid and the outer membrane together (26). Determining the exact functions of the tegument and its constituent proteins has been made very difficult for a number of reasons. First, the tegument itself, while containing conserved and critical protein components, is diverse in both structure and composition (8, 24). Furthermore, a variety of studies have shown that there is redundancy between different components of the tegument, making it difficult to elucidate their specific roles and whether they are absolutely required (24). It is known that there are two layers to the tegument—the inner layer, closest to the capsid, and the outer layer, which is adjacent to the outer membrane. Each of these layers contains specific proteins that are involved in a number of different functions.

Finally, the outermost portion of a mature herpes virion is the envelope (Figure 3a). This structure is derived from a host cellular lipid bilayer membrane, and contains both viral and host proteins (24). The herpesvirus envelope is believed to potentially originate from the trans-Golgi network or related structures, such as ER-Golgi intermediates, depending on the virus (26). The most critical function of the envelope is recognition and attachment of the virion to the appropriate host cells via viral-encoded glycoproteins (24). However, the envelope also makes the virus more susceptible to inactivation via disruption of the lipid bilayer membrane.

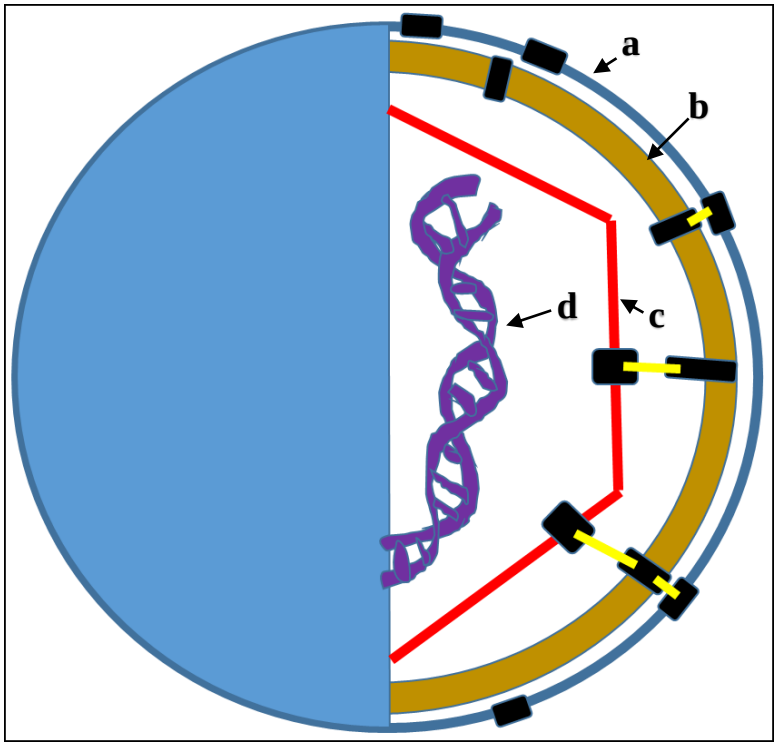

Figure 3 The structure of the herpes virion. There are various membrane-bound and transmembrane proteins that bind each layer to the other, as denoted by the black boxes connected by yellow lines. a) The outermost layer, the lipid envelope, is derived from the host cell. b) The layer deep to the lipid envelope is the proteinaceous tegument. While the functions of some of the proteins found here are known, many are not. c) Deep to the tegument is the capsid, comprised of a specific pattern of protein complexes. d) Within the capsid resides the viral DNA (much more DNA is packed into the capsid than pictured).

Life Cycle of Herpesviruses

I will focus my discussion on the productive lytic cycle of herpes virus replication as this is the cycle that is targeted by antivirals and for which the best information exists. Although the

discussion will focus on aspects of HSV-1 productive replication as a general model, when appropriate, homologous functions of other herpesviruses will be noted.

For a virion to successfully infect a host cell and produce progeny, it must first recognize and attach to a receptor present on the plasma membrane of the host cell (Figure 5a). This attachment is mediated by glycoprotein components of the virion envelope. These glycoproteins, including gB, gC, and gD in HSV-1, function in recognizing specific host cell receptors (24). The glycoprotein composition of each herpesvirus and the specific cognate cellular receptors determine both the host and tissue tropism of each virus. Following attachment of the virion, some of the viral glycoproteins mediate fusion of the viral envelope and plasma membrane for entry of the capsid and associated tegument proteins (24). For HSV-1, alternate methods of entry involving pH-dependent entry via endocytic pathways also occur, though the specific method of entry depends on the cell type being infected (24). Following entry of the capsid and at least some of the tegument proteins into the cytoplasm of the host, many of the tegument proteins dissociate from the capsid due to their phosphorylation by viral and/or cellular kinases (8, 24). One of these kinases, pUL13, is conserved in the *Herpesviridae* family, and may play a similar role in the other herpesviruses (24).

The subset of the inner tegument proteins that remains attached to the capsid may perform certain key functions required in getting the viral capsid and its genetic payload to the nucleus (Figure 5b) (24). It has been proposed that some of the tegument proteins function in linking the capsid to the dynein/microtubule transport system, as evidenced by the physical association between inner tegument pUL35 and dynein (24). Two of the inner tegument proteins that remain associated with the capsid are pUL36 and pUL37, which are also conserved across *Herpesviridae*, suggesting a potential role in trafficking of the capsid (24). No specific

interactions between pUL36 or pUL37 and dynein have been identified, however, so they may be involved in other functions (24). Some important differences in the apparent functions of these proteins or their homologs do exist between different viruses. For example, the HCMV pUL37 homolog, pUL47, is not necessary for entry and trafficking of the capsid to the nucleus (24). In addition to a putative role in binding the dynein transport system, pUL36 is also believed to function in targeting the capsid to the nucleus, potentially by interacting in some way with the nuclear pore complex (24). pUL36 has been shown to have a nuclear localization signal sequence, consistent with a role in directing the capsid to the nucleus (24). It has also been noted that cleavage of the N-terminal end of pUL36 is followed by entry of the viral DNA into the nucleus, suggesting that pUL36 may play a role in injection of viral DNA into the host nuclear compartment (24). A second key protein believed to play a role in releasing the viral DNA from the capsid is pUL25, which may interact with the nuclear pore complex (8, 26).

Following entry of the viral DNA into the nucleus, several events must occur. These include transcription of viral genes, synthesis of viral proteins from these transcripts, replication of the viral DNA to amplify the genomes for packaging into capsids, and the beginning of virus capsid assembly (Figure 5c, d, e). Several components of both the virus and host cell are utilized in completing this process, which must occur before egress from the nucleus and maturation of the virion particle. Like many developmental systems, there is a pattern that emerges in the temporal regulation of herpes virus gene expression (24). This regulation occurs coordinately and in a cascade fashion, starting with immediate-early gene expression to set the stage for replication (Figure 4) (24). These immediate-early genes play primarily regulatory roles that serve to enhance viral gene expression and do not require the prior expression of viral protein (24). Some (such as ICP4) are required by the subsequent wave of transcription of early genes,

many of which are required for the process of DNA replication and for the regulation of cell functions that are required for replication or which need to be subverted to permit replication or prevent cell death (24). The last wave of gene expression leads to the synthesis of a variety of structural components of the virus (e.g. capsid proteins, envelope proteins, tegument proteins) as well as proteins that are required for the processing of replicated DNA and its insertion into empty capsids and their maturation (24).

Both tegument and capsid proteins have been shown to play a role in the regulation of gene expression, though they are not conserved across all herpesviruses, as some are only present in specific subfamilies (e.g. pUL48 in *Alphaherpesvirinae* members) (24). Expression of the early genes leads to the production of factors that initiate replication of the viral DNA as well as the expression of late genes, and which are also not conserved across all *Herpesviridae* family members (24). In addition to acting as regulators of transcription, some tegument proteins function at the level of regulating translation, either preventing the general synthesis of protein in the host (e.g. pUL41 endoribonuclease activity degrading both host and viral mRNA) or preventing host mechanisms from shutting down gene expression (e.g. ICP0 targeting cellular proteins such as interferon for degradation via ubiquitin ligase activity) (24). Still other tegument proteins play a role in replication of viral DNA, such as thymidine kinase (pUL23) and dUTPase (pUL50) which function in nucleotide metabolism in non-dividing host cells (24).

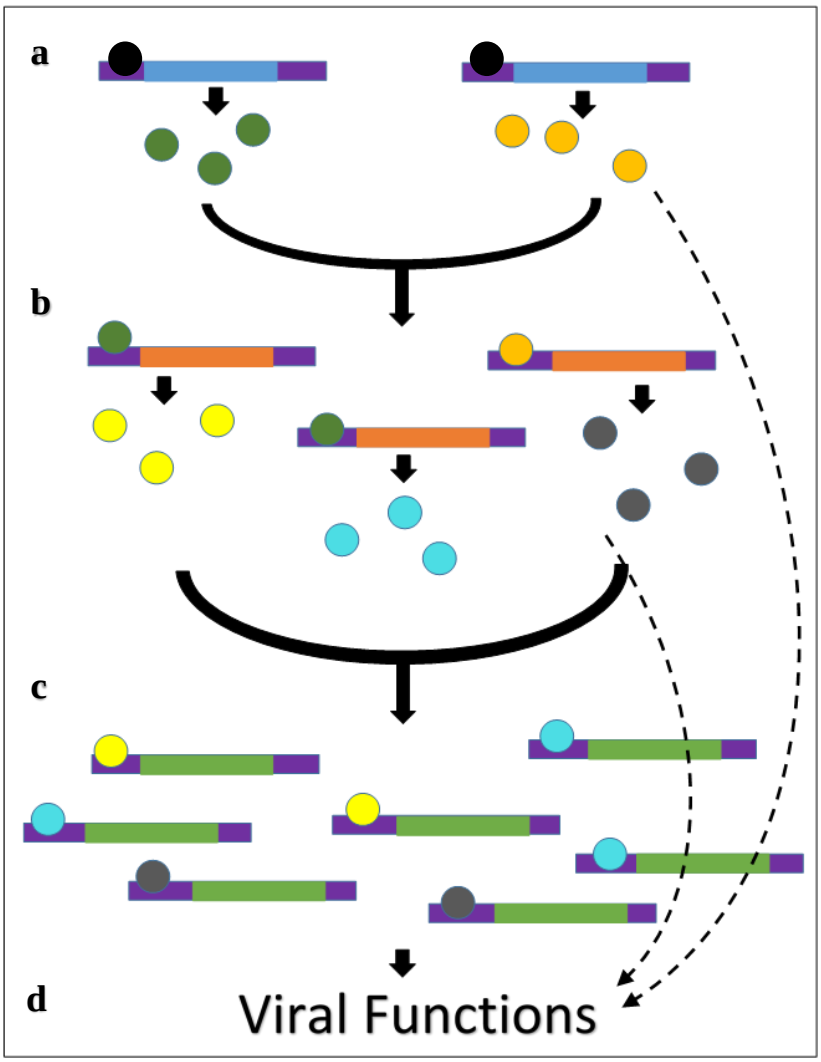

Figure 4 Overview of cascade expression in herpes viruses. These waves are often coordinated with each other and with the environment/host. This coordination can be regulated by protein interactions, signals, and other regulatory mechanisms. a) Initially, the first wave of genes (the immediate-early genes, in pale blue) will be expressed without any prior viral synthesis via proteins included in the capsid (at least in the case of HSV-1) (24). b) A second wave of gene transcription is triggered by the products of the first wave. These represent the early genes (in orange) in the herpes virus cascade. c) A third wave can be triggered by the products of the second wave. In herpes, these are the late genes (in green). d) Different protein products will play different roles in the life cycle of the virus.

Herpesvirus DNA Replication

Shortly after the HSV-1 genome enters into the nucleus, the linear DNA circularizes, likely using host machinery (28, 40). DNA replication is carried out by a complex of proteins referred to as the replisome. Though other proteins are likely to be present, the replisome contains six core viral-encoded proteins that are conserved across the *Herpesviridae* family (28,

40). The HSV-1 DNA polymerase holoenzyme is a heterodimer composed of two subunits, pUL30 (catalytic subunit) and pUL42 (processivity subunit) (40). A heterotrimer comprises the helicase-primase complex, and is made up of proteins pUL5, pUL8, and pUL52 (28). The final core protein is a single strand DNA binding protein designated as pUL29/ICP8 (40). There are additional proteins that play important and even, essential, roles in DNA replication that are not conserved across all herpesviruses. One of these proteins (for HSV-1 and HSV-2) is the origin binding protein (OBP), encoded by UL9 (28, 40). pUL9 binds specifically to regions of DNA that are known sites where DNA replication is initiated (origins) (28, 40). HSV has 3 bona fide origins, though only 1 is necessary for DNA replication, at least in cell culture (40). In the most accepted model for initiation of viral DNA replication, pUL9 both binds to and initiates DNA replication from origins, presumably by opening the duplex DNA using its helicase activity, and by permitting entry of the other replisome proteins through its ability to physically interact with the members of the aforementioned subcomplexes (28, 40). Activation of replication for certain other herpesviruses, such as HCMV and KSHV, is more complicated than for HSV-1 or HSV-2, and does not require the product of pUL9 or an ortholog (40). For example, it is thought that transcriptional transactivators may be involved in initiation of DNA synthesis for some herpesviruses (40).

For HSV-1, replication occurs in defined locations within the nucleus, known initially as foci, which develop as time progresses into sites of localized replication, also known as replication compartments (28). The first step that must occur is activation of one of the three origins of replication present in the genome of HSV-1, which is carried out by pUL9 (OBP) and ICP8 (28, 40). These proteins bind to one or more of the origins and promote conformational changes in the DNA that cause the two strands to unwind at a defined location, allowing the

replication proteins access to the DNA (28, 40). It should be noted that this series of steps has been identified in an *in vitro* system, where a number of questions remain regarding the specific conditions in an infected cell and how synthesis is initiated in such an environment (28, 40). Following activation of the origin, the helicase/primase complex is recruited to the DNA, where it functions as its name suggests—the double stranded DNA is unwound and RNA primers are laid down in preparation for replication (40). Recent findings demonstrate that this complex may not exist simply as a trimer, but rather in some higher order structure that is important in the process of DNA replication (28, 40). It is worth noting that pUL8 has no recognized cellular homolog protein, and that the complex itself can be targeted by antivirals (28). Finally, the DNA polymerase complex is recruited to the DNA, likely through some conformational change in the higher order structure of the helicase/primase complex or by the presence of primer on the single stranded DNA (40). Thus far, no one has managed to recreate origin-dependent synthesis of viral DNA outside of cells, so the exact process that occurs intracellularly remains unclear (40). Replication of both the leading and lagging strands occur in the initial stages of replication, which is thought to occur via a theta-replication mode (28). At later times, a second mode of replication is thought to ensue (via a rolling circle mode) which leads to the generation of long, head-to-tail concatemers (28). These concatemers must be processed to unit-length DNA genomes with specific ends that are packaged into nascent assembled, but empty capsids.

In addition to proteins that are directly involved in DNA synthesis, herpesviruses encode additional proteins, known as auxiliary proteins, which support virus replication, but are so named because they are not absolutely essential for viable virus production, at least in tissue culture or in some cell types (40). These proteins include thymidine kinase (pUL23), which functions in nucleotide metabolism by phosphorylating nucleosides (40). Another important

auxiliary protein is the nuclease pUL12, which contains 5'-3' exonuclease activity and is required for replication of infectious virus *in vivo* (40). It should be noted that the alkaline nuclease is conserved across all herpes viruses, and its homolog in HCMV is designated pUL98 (6, 18, 25). These auxiliary proteins have also been identified as targets for the development of antiviral drugs.

Capsid Assembly

The final steps in the lytic life cycle are assembly and processing of virus structural components, maturation of the virion, and egress of the virus from the host cell (Figure 5f, g, h). The capsid is assembled inside of the nucleus and DNA is inserted into empty capsids in a coordinated fashion that involves interactions among concatemeric DNA, a complex of proteins called the terminase, and the unique vertex on virus capsids known as the portal (21). The process may have evolved from similar processes in certain bacteriophage (26). The major components of the capsid, pUL19, pUL18, and pUL38, are assembled into empty or pro-capsids and contain an inner scaffolding protein, pUL26, required for the DNA to be inserted (8, 26). It is proposed, but not proven, that the dodecameric portal complex, composed of pUL6 subunits, serves as a nucleation site for the auto-assembly of the procapsid (8, 21, 26). Following formation of the procapsid, pUL26, an autocatalytic protease, initiates cleavage of portions of the scaffold as the viral DNA is being packaged (8, 26).

Several proteins are required for the proper packaging of viral DNA, many of which are conserved across the herpesviruses, including pUL15, pUL28, and pUL33, which form the terminase complex, and pUL17 and pUL32 (26). Interestingly, pUL17 helps to recruit pUL25 to the capsid, where both of these proteins may be considered the beginning of the inner tegument, rather than capsid proteins (26). These proteins form a heterodimer that is believed to function in

stabilizing the capsid through their interactions with each of the capsid vertices (21). The function of pUL32 has not been fully identified, but it is thought to potentially play a role in proper localization of the capsid so that DNA packaging can occur (21). Again, the ATPase subunit of terminase, pUL15, is the most conserved gene in *Herpesviridae*, indicating a critical role in the life cycle of human herpesviruses (21). *In vivo*, pUL15 forms a heterotrimer with pUL28 and pUL33, to form the functional terminase complex (21, 38, 45). The complex forms in the cytoplasm and is subsequently localized to the nucleus of the host via a NLS encoded by pUL15 of HSV-1, while the terminase homologs in CMV require oligomerization for efficient localization (21, 38). Terminase is critical in the actual process of DNA packaging, as it forms a machine capable of inserting DNA into the capsid, despite the unfavorable interactions caused by tight DNA packing (21). Terminase functions in recognizing unit length genomes in the concatemeric DNA that is formed during viral replication and in cleaving the DNA at specific signals to yield defined ends (45). Due to the difficulty of purification of the intact terminase complex because of its insolubility, the exact functions of each of the subunits is only beginning to be elucidated (21). As demonstrated by both Yang et al and Heming et al, mutations in key regions of HSV-1 pUL15 and pUL28 lead to loss of significant viral replication and improper DNA packaging and cleavage (21, 45). The role of pUL33 is not as clear, though it is believed to play a role in promoting the interaction between pUL15 and pUL28 (21, 38). The analogous genes in human cytomegalovirus are believed to be pUL89, pUL56, and pUL51 (pUL15, pUL28, and pUL33 in HSV-1, respectively) which are somewhat conserved in function (though not as much in sequence) but subtly different in some of their characteristics and interactions (38). As the DNA is packaged into empty pro-capsids (also known as B capsids), the autoproteolytic pUL26 scaffolding protein is cleaved. If the process is completed successfully with the

terminase cleaving the appropriate DNA end and "head-full" amount, the procapsid will undergo a conformational change to produce electron-dense complete capsids (also called C capsids). If the process does not occur properly, the DNA will be exuded, leaving an empty capsid that lacks detectable intact scaffold (also called A capsids) (8, 45).

Virion Maturation and Egress

To complete the entire replication cycle, the capsid must exit the nucleus and mature into an infectious virion containing tegument and an envelope (Figure 5f, g). The generally accepted model of egress is known as the envelopment-deenvelopment-reenvelopment model (24, 26). In this model, the nucleocapsid exits the nucleus by budding through the inner lamellae of the nucleus. This enveloped nucleocapsid is not infectious, but subsequently fuses with the outer lamellae of the nucleus, forming the envelopment-de-envelopment steps of the model (8, 24, 26). The pUL17/pUL25 complex has been shown to be necessary for proper egress from the nucleus, and it is believed that pUL25 mediates interactions between the capsid and the inner membrane (8, 26). Whether or not pUL25 binds to other proposed nuclear egress proteins, such as pUL31 and pUL34, is not clear (8, 26). However, the pUL31/pUL34 complex has been shown to be necessary for proper nucleocapsid budding (24, 26). The proper localization of the pUL31/pUL34 complex has been indicated as a role for the viral kinase pUS3, which also functions in phosphorylating the nuclear lamins A/C (24). Deletion studies show that the pUL31/pUL34 complex forms inappropriate aggregations in the absence of pUS3 (24).

Once in the perinuclear space, the enveloped nucleocapsid must fuse with the outer nuclear membrane to gain access to the cytoplasm of the host cell. It has been shown that pUS3 is required for efficient movement of the particle from the perinuclear space to the cytoplasm, as loss of this kinase leads to accumulation of enveloped nucleocapsids between the outer and inner

nuclear membranes (24, 26). In addition, glycoproteins gB and gH, which are used in entry of the mature virus into host cells, have been implicated in playing a role in nuclear egress, though the specifics are not clear and much disagreement exists (24, 26). Studies have shown that nuclear egress and viral entry remain separate processes, and that the use of glycoproteins in exiting the nucleus may represent a virus or sub-family specific phenomenon (26).

The last steps include finishing tegumentation of the nucleocapsid and secondary envelopment (reenvelopment portion of the egress model), which occur in the cytoplasm of the host (Figure 5g). Some tegument proteins are thought to potentially be added prior to this step, while the capsid is still in the nucleus, though the data is contradictory (24). Regardless, it is at this point that the inner tegument proteins pUL36 and pUL37 are added to the nucleocapsid, as well as other proteins that may be specific to a particular herpes virus or to sub-family members (24, 26). The pUL36 protein is likely to be recruited via interactions with pUL25, which in turn recruits pUL37 (24, 26). Thus, it is proposed that pUL36 acts as a sort of "nucleation" point for the addition of inner tegument proteins, serving as a recruitment factor (26). Localization studies indicate that these proteins are added to the nucleocapsid at or near the Golgi (24). In addition to recruiting other tegument proteins, pUL36 is necessary for transport of the capsid to the correct cellular location for secondary envelopment (24, 26). Finally, pUL36 is proposed to play a role in linking the inner and outer tegument layers along with pUL48, contributing to the stable formation of mature virus (24, 26). Whereas the inner tegument is largely specific in the relative amounts of its constituent proteins, the outer tegument is much more variable, suggesting significant redundancy in function between many of the protein subunits (24, 26). A trimer composed of pUL11, pUL16, and pUL21 is believed to play a role in anchoring the tegument to the viral envelope, though the necessity of this complex varies among herpes viruses (24, 26).

Paralleling the ability of some tegument proteins to recruit other tegument proteins is the ability of the tails of some glycoproteins to recruit outer tegument proteins (26). Thus, addition of tegument proteins and maturation of the virion is a complex and multistep process. Many other studies have shown a variety of defects in packaging and maturation of the viral particles when various putative glycoproteins and tegument proteins are mutated or missing, but the exact function of these constituents and their addition to the virion is incomplete and lacking in detail (26).

Prior to release of mature virus from the host cell, the maturing nucleocapsid with associated tegument must acquire its final envelope (Figure 5g). Secondary envelopment likely occurs either within the trans-Golgi network or in an ER-TGN intermediate (26). The nucleocapsid, together with its tegument proteins, are thought to obtain their final envelope from TGN membranes containing viral glycoproteins (26). These forms are infectious and may be released into the cytoplasm in secretory vesicles capable of fusing with the plasma membrane to release the fully mature, infectious enveloped virus particle (Figure 5h) (26).

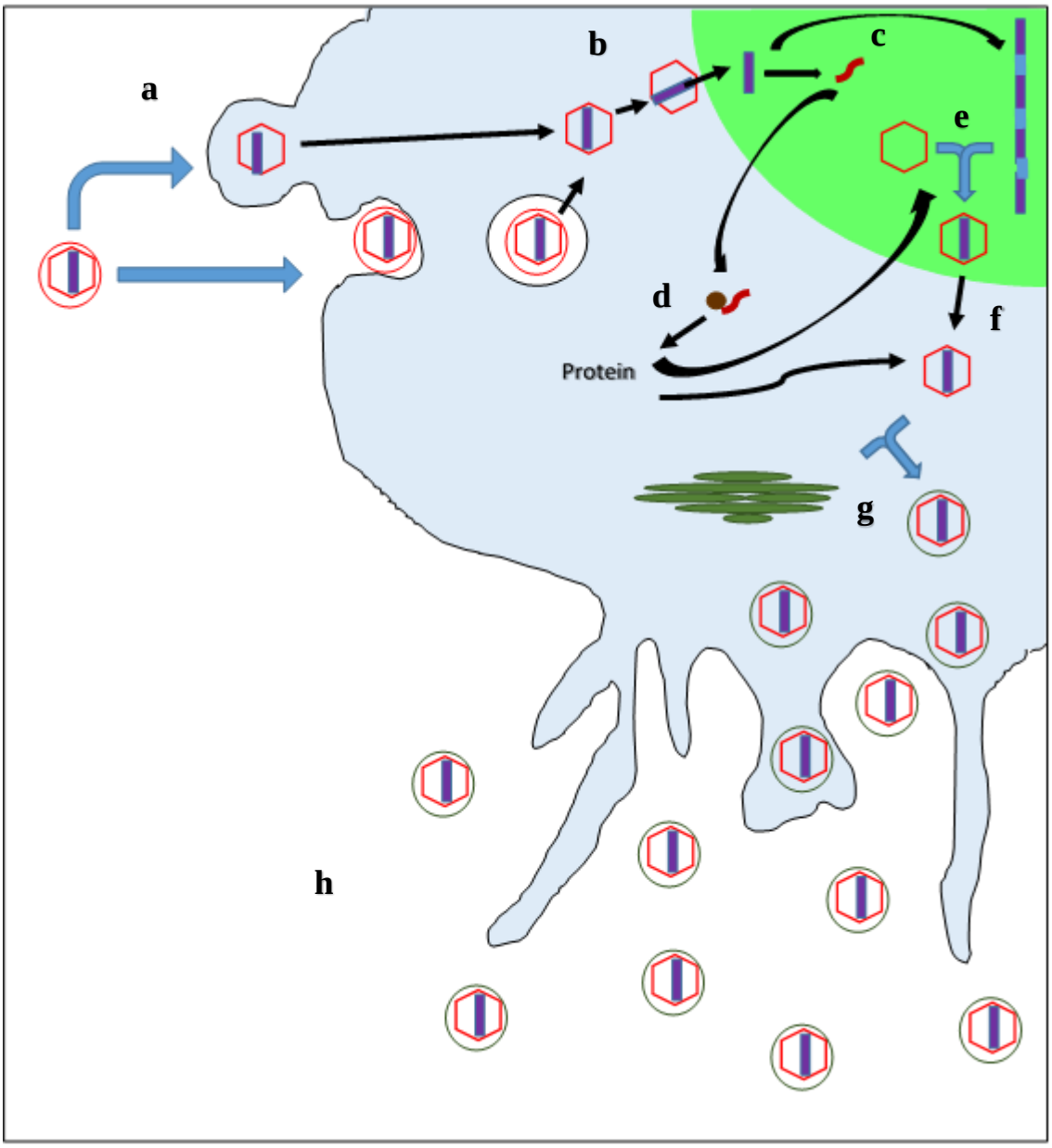

Figure 5 Summary of the lytic cycle for herpes viruses. a) The virion recognizes a host cell and can either fuse with the plasma membrane of the cell or be taken in via endocytosis mediated entry. b) Once inside the host, the capsid is transported to the nuclear membrane (the nucleus is in green), through which the viral DNA is injected. c) Once inside the nucleus, the viral DNA initiates cascade replication via the immediate-early genes. Subsequent expression produces mRNA that is transported to the cytoplasm. Concurrently, long head-to-tail concatamers of viral DNA are produced, which must be processed and properly packaged to produce fully mature capsids. d) Viral mRNA is produced in large amounts, siphoning ribosomes away from host translation. e) Some of the viral proteins are transported back into the nucleus, where they may act in a variety of ways. This includes roles as transcription factors, components of the capsid, and components of the viral replication and packaging machinery. f) After packaging, the mature capsid egresses into the cytoplasm. g) Further virion maturation occurs within the cytoplasm, where tegumentation is completed and envelopment occurs (believed to be derived from the Golgi apparatus). h) Viral replication and production continues until the host cell is compromised. Lysis of the cell releases the virions, and the process begins anew.

Human Cytomegalovirus

At this point, I will now shift the focus of discussion from information about general herpesviruses to the prototypical member of *Betaherpesvirinae* and the subject of my research, CMV (27, 32). With a worldwide distribution, CMV is a ubiquitous pathogen that has a prevalence in human adult populations of anywhere between 40 and 100%, depending on geographic location, socio-economic status, age, sexual activity, and other related factors (32, 33, 37). As with all herpesviruses, CMV establishes a latent infection following primary infection, and is capable of periodic reactivation. Latency is established within cells of the myeloid lineage, where CMV is present as an episome, an extrachromosomal piece of circular DNA that is maintained and persists throughout the lineage of its host cell (16, 33, 37). It has also been shown that CMV can be detected in the endothelial cells and epithelium of patients with active infection and that the virus can be grown in these cell types *in vitro*, though latency in these cell types has not been demonstrated (32, 33). Transmission is made possible following activation of the lytic life cycle through the shedding of virus in a variety of body fluids, including breast milk, semen, saliva, urine, cervical secretions, placental transfer, blood transfusions, and organs (through organ transplant) (33, 34). Close contact between individuals is generally required for transmission of the virus, usually from an asymptomatic individual who is actively shedding virus (16).

The outcome of primary infection, reinfection, or reactivation is largely dependent on the status of the host individual—those that are immunocompetent are often either asymptomatic or mildly symptomatic while shedding virus, whereas those persons who are immunosuppressed can suffer a variety of diseases and pathologies (16, 27, 32, 34, 37). CMV is particularly associated with a reduction in and functional depression of T cells in host individuals (16, 33).

Thus, those persons who are infected with HIV and have AIDS, or individuals who are undergoing immune suppression (i.e. transplant recipients, cancer patients) are especially at risk of severe disease. The diseases that are associated with CMV active infection are numerous, devastating if untreated, and sometimes fatal. Some of these include pneumonitis, retinitis, gastritis, and colitis (33, 34). Some diseases are more prominent in different patient subpopulations. Retinitis makes up the vast majority of the manifestation of CMV infection in HIV patients (34). However, pneumonitis is exceedingly rare in AIDS patients coinfected with CMV, likely due to an interaction between the host's immune system and CMV (33). In addition, CMV is the most prevalent and important congenital infection in developed countries, causing significant diseases and birth defects in neonates (7, 32, 36, 37). Among these are hepatosplenomegaly, jaundice, petechiae, microcephaly, mental retardation, and sensorineural impairment affecting vision and hearing (7).

There are a number of other manifestations that have been shown to be associated with CMV but have not been confirmed to be caused by or a direct consequence of CMV. Many of these manifestations are believed to operate through host immune system interactions with CMV—manifestations which include autoimmune diseases such as Guillain-Barre syndrome, type I diabetes, systemic sclerosis, organ transplant rejection, and vascular disease (33, 34). CMV has a dynamic interaction with the immune system of its host, and contains many genes that have been shown to interfere with and alter the host immune response in vitro (7, 33, 37). Annotation of the CMV genome has either confirmed (19 genes) or led to the identification (14 genes) of a number of different genes that interact with the immune system of the host in some way (37). Prominently, CMV interferes with antigen processing in immune cells by inhibiting the presentation of MHC class I molecules through the activity of a number of gene products

found in the unique short region of its genome (7, 33, 37). It is believed that CMV may also play a role in the down regulation of MHC class II genes in a similar manner (7, 33). Other interactions between CMV and host immune components have been characterized to lesser degrees, including various interactions with natural killer cells and dendritic cells, and the production of various small, chemokine-like molecules (7, 33, 37). These complicated interactions and pathways are proposed to play significant roles in the development of some of the autoimmune manifestations associated with CMV (33).

More recently, CMV has been implicated in oncomodulatory roles in infected individuals, particularly those that are immunocompromised (13, 27). However, the precise role that CMV plays is debated, and no real consensus has been reached. Some studies purport that CMV infection is cancer protective while others find no significant association between CMV and cancer progression (13, 27). Regardless, enough evidence exists that CMV has been examined as an agent in cancer development for several decades (27). While CMV cannot be regarded as a tumor virus, due to the complete absence of evidence indicating its ability to transform normal host cells, there are a number of associations that have been observed between CMV and cancer (13, 27). Antigens and nucleic acids belonging to CMV have been found within tumor tissues removed from patients, though the association has yet to be explained (13, 27). A recent study shows a correlation between active CMV infection and the onset of gastric cancer, though CMV as a causative agent of gastric cancers has remained hypothetical (13). The reservoir of data regarding the roles that CMV may play in cancer development and progression continue to be expanded, and are an important area of research.

Antiviral Drugs that Combat Herpesviruses

Due to their significant impact on human health, herpesviruses are one of the three major categories of virus for which there are available, targeted antiviral drugs (30). Since the 1960s, nucleoside analogues have served as the compound of choice for anti-herpesvirus drugs to viral replication and spread (14, 30). Currently, nucleoside analogues make up the vast majority of available herpesvirus antivirals, though there are some limited alternative drugs available. Additionally, there exists an effective vaccine for VZV that is used both in children and the elderly, populations that are at risk for chickenpox and herpes zoster, respectively (15). Vaccines for other human herpesviruses, however, are lacking; little success has been made with regards to vaccines against HSV (15). Some progress has been made with two different variants of a CMV vaccine, one of which contains viral proteins and the other which contains DNA plasmids that can express CMV genes (15). As for EBV, HHV-6, HHV-7, and KSHV, no vaccines are currently available (15).

All of the currently approved antiherpes drugs used in the United States act by inhibiting replication of the virus, specifically by inhibiting the viral DNA polymerase (Table 1) (15, 30, 41). Different compounds have varying efficacies against the different human herpesviruses. Acyclovir, penciclovir, and foscarnet are generally used against the alpha herpesviruses HSV-1, HSV-2, and VZV (15, 30, 41), while ganciclovir, cidofovir, and foscarnet are used to treat CMV (15, 19, 23, 30, 41). Currently, there exists no antiviral treatments for EBV, HHV-6, HHV-7, or KSHV (41). Valacyclovir, famciclovir, and valgancyclovir are the oral prodrug variants of acyclovir, penciclovir, and ganciclovir, respectively (15, 19, 23, 30, 41). These variants have significantly higher bioavailability via the oral route than their non-modified counterparts (15, 30). Once in the body, the prodrug variants are converted back into their respective base

compound by host metabolic processes (16, 30). Thus, the mechanism of action for the prodrugs is the same as their base variants.

Many of the antiherpes drugs share very similar mechanisms of action, though key differences exist for some of the compounds (Table 1). Acyclovir, penciclovir, and ganciclovir are all nucleoside analogues that must be converted into an active form by a combination of viral and cellular enzymes (15, 19, 23, 30, 41). In each case, the inactive drug must be initially phosphorylated by a virus-encoded kinase, thymidine kinase from HSV in the cases of acyclovir and penciclovir, and a protein kinase (pUL97 from CMV) in the case of ganciclovir (15, 19, 23, 30, 41). The monophosphate forms of these compounds are then further modified by the cellular machinery of the host to produce the active triphosphate forms of the drugs (15, 19, 23, 30, 41). The active forms then function in inhibiting viral DNA replication by competitively binding to the active site of the viral DNA polymerase and terminating DNA elongation upon incorporation into the growing chain (15, 19, 23, 30, 41). Cidofovir has a bound phosphate group, thus eliminating the need for activation (formation of the monophosphate) via viral kinase activity (19, 23, 30). This reduces the specificity of the drug's action and results in the severe toxicity of the drug in humans. Foscarnet is unique when compared to the other antiherpes drugs in that it is a non-nucleoside pyrophosphate analogue (19, 23, 30). Unlike the other compounds, foscarnet does not need to be activated via phosphorylation, instead inhibiting the viral DNA polymerase by competitively binding its pyrophosphate binding site and preventing the binding of incoming dNTPs (19, 23, 30).

Acyclovir and penciclovir, the antivirals of choice for HSV-1 and HSV-2 infections, have a number of qualities that makes them particularly effective (15, 41). While all of the antiherpes drugs have some degree of toxicity, these two drugs have a well-established safety record (41).

Some of the more common adverse effects caused by these drugs include low rates of reversible nephrotoxicity, reversible neurological issues, and nausea (30). In general, the nucleoside analogues are safer and have lower toxicities than the other drugs due to several factors, including the necessity of a viral kinase for activation and poor inhibition of host DNA polymerases (15, 41). Additionally, the frequency of clinically relevant mutants resistant to these antivirals has remained relatively low at roughly 0.5% (15, 41). Though resistance does occur, it often results in a significant reduction of the virus to be able to replicate, causing it to become nonpathogenic (41). Current treatments for CMV, however, do not share these beneficial features, indicating a need for the continued development of new antiviral therapies. Due to the nature of the antivirals and their side effects, none of them are approved for treatment of congenital CMV infection (1, 2). Cidofovir (a nucleotide analog) has a higher toxicity than the nucleoside analogues, causing nephrotoxicity and myelosuppression (23, 30). Similarly, foscarnet is associated with significant toxicity, causing nephrotoxicity in 30% of patients, as well as electrolyte disturbances and neurological side effects (1, 30). The increased toxicity of these compounds is related to the fact that they do not need to be activated by viral protein, instead requiring only host proteins in the case of cidofovir or being functional without activation as with foscarnet (16). The drug of choice for combating CMV, ganciclovir, is associated with a number of toxic effects on the host. Most commonly, treatment with ganciclovir results in myelosuppression, though a number of other effects are possible including CNS toxicity, nephrotoxicity, gastrointestinal distress, and abnormal liver tests (1, 30). Clinical data shows that roughly 10% of patients must stop treatment, and that approximately 33% of patients must have their dosage adjusted, interrupted or discontinued (1, 41).

Unlike acyclovir and penciclovir, resistance of the target virus to ganciclovir occurs much more frequently (41). The development of ganciclovir resistant CMV occurs most commonly in individuals being treated with ganciclovir over a long period of time, as is the case with immunosuppressed individuals (1, 19, 23). Resistance occurs from mutations in either pUL97, the viral kinase, or pUL54, the viral DNA polymerase (1, 19, 23). Mutations in pUL97 confer resistance to ganciclovir, as it is needed to catalyze the initial phosphorylation step, whereas mutations in pUL54 can confer resistance to ganciclovir, cidofovir, foscarnet, or some combination of these drugs (19). Studies have shown that resistance to ganciclovir and foscarnet develops in 13-27% and 37%, respectively, of patients exposed to the drugs for one year, with cidofovir believed to have similar resistance rates (19). If resistance does occur in a patient, current treatments include increased doses of ganciclovir or a combination of ganciclovir and either cidofovir or foscarnet, all of which are associated with significantly increased toxicity (19, 23).

The toxicity of current antiviral treatments for CMV coupled with increasing numbers of resistant strains have led to the investigation of a number of new compounds. One of these compounds is known as CMX001, an oral prodrug variant of cidofovir (1, 15, 19, 23). Having completed stage II clinical trials, CMX001 shows greater activity against CMV with a less severe toxicity profile (15, 23). Maribavir is an inhibitor of pUL97 that showed promise, progressing to phase III clinical trials (1, 19, 23). However, despite its increased safety relative to current treatments, maribavir failed the phase III trials due to lack of efficacy (1, 23). These two compounds still target either pUL97 or pUL54. Thus, although they are safer than ganciclovir, GCV-resistant mutations in these genes often result in resistance to these compounds as well. Therefore compounds that affect novel targets are needed. A few have been identified and are

currently being investigated. Letermovir, tomeglovir, and BDCRB are all compounds that inhibit viral replication by interfering with the terminase enzyme, the key component for proper packaging and cleavage of replicated DNA, and a target that is truly unique to viruses (15, 19). The need for additional antiviral drugs to combat CMV is still great, particularly those that function against novel and virus-specific targets. Though some compounds are being investigated for inhibition of terminase function, other targets exist that have the potential to be specific, safe, and effective.

Antivirals	Targets	Some Common Side Effects	Primary Use:	Notes
Acyclovir/ Valacyclovir (oral prodrug)	Viral DNA polymerase-- nucleotide binding site; requires monophosphorylation by viral enzyme	Nephrotoxicity, neurological complications, nausea	HSV-1, HSV-2, VZV	Established safety record; reversible side effects; relatively low toxicity; low rates of clinically relevant resistant mutants
Penciclovir/ Famciclovir (oral prodrug)	Viral DNA polymerase-- nucleotide binding site; requires monophosphorylation by viral enzyme	Nephrotoxicity, neurological complications, nausea	HSV-1, HSV-2, VZV	Established safety record; reversible side effects; relatively low toxicity; low rates of clinically relevant resistant mutants
Ganciclovir/ Valganciclovir (oral prodrug)	Viral DNA polymerase-- nucleotide binding site; requires monophosphorylation by viral enzyme	Myelosuppression, CNS toxicity, gastrointestinal distress, abnormal liver tests	CMV	Higher toxicity than acyclovir and penciclovir; high rates of resistant mutants
Cidofovir	Viral DNA polymerase-- nucleotide binding site	Nephrotoxicity, myelosuppression	CMV	Does not require activation by viral enzyme; significant toxicity
Foscarnet	Viral DNA polymerase-- pyrophosphate binding site	Nephrotoxicity, neurological complications, electrolyte disturbances	HSV-1, HSV-2, VZV, CMV	Active without activation by viral enzyme; significant toxicity

Table 1 Overview and summary of the antivirals used to combat herpes viruses.

Alkaline Nucleases

One such target for novel antivirals is the herpesvirus alkaline nuclease. Conserved across the *Herpesviridae* family, the nuclease is a member of the λ exo DNase family of proteins, within the PD-(D/E)XK superfamily of DNA-modifying enzymes (6, 17, 25, 31). Elucidation of

the evolutionary relationship between various enzymes within this superfamily shows that phage and virus nucleases originate from a single ancestor (6). Subsequent divergence and host-specific evolution has led to a low level of sequence similarity between the various proteins in this family (6). However, conservation of the putative active sites of these enzymes has allowed their relationships to be deduced despite differences in their presumed structure (6). These differences in structure give the various enzymes their substrate specificities, as evidenced by the necessity of particular N and C-terminal domains in the HSV and EBV nucleases that allow them to bind to DNA, which are absent in the λ nuclease (6).

Despite years of study and knowledge of its evolutionary history, the exact *in vivo* role(s) of the herpesvirus nuclease remain unknown, though its conservation in *Herpesviridae* provides one reason to believe that it serves an important role (18). *In vitro*, the nucleases have both 5'-3' exonuclease and endonuclease activities, with the exo activity capable of degrading both single and double-stranded DNA (20, 25, 29, 31, 42). These enzymes exhibit a high pH optimum (alkaline), require the binding of a divalent metal cation (Mg^{2+}), do not require ATP, and are inactivated at high concentrations of salt (20, 25, 29, 31, 42). Several studies using pUL12, the HSV-1 alkaline nuclease, have been performed, providing an indication of the role that the alkaline nucleases may play *in vivo*. It is known that pUL12 localizes to the nucleus of the host cell and that it is present at early times following infection (20). Resolution of HSV-1 replication intermediates by electrophoresis following digestion with restriction enzyme shows that in pUL12 null mutants, the DNA intermediate is even more complex than the wild type intermediates (20). Additionally, lacZ insertional mutants, which inactivate pUL12, lead to a significant decrease in plaque formation with only a small amount of infectious virus production (42). However, these mutants show little reduction in DNA or protein synthesis, indicating that

pUL12 is in some way required for efficient virion production but is not necessary for viral macromolecular synthesis (42). Further investigation of the role of pUL12 revealed that it is the loss of nuclease activity, specifically exo activity, which resulted in the inability of mutant variants of UL12 in plasmids/amplicons to complement null mutants and restore wild type viral production in cell culture (20, 22). Continuing along this line of thought, a recent study characterized the role of the nuclease activities of pUL12 with regards to viral replication and neurovirulence using recombinant virus (17). It was discovered that the nuclease activity of pUL12 was not actually required for replication of the virus in cell culture, but that it is necessary for virulence in a mouse model (17). Thus, there may exist another function for pUL12, independent of its nuclease function, which is critical in the life cycle of HSV-1 (17). Other experiments have shown that pUL12 associates with ICP8 and that in combination these proteins can catalyze strand-exchange *in vitro* (29). This led to an exploration of the possibility that pUL12 may function in some sort of recombination pathway, though subsequent studies showed that pUL12 is not necessary for neither intra- nor intermolecular recombination (29).

In summary, decades of research, while unable to elucidate the actual role that pUL12 plays in the HSV-1 infection cycle, have revealed many facets to this enzyme. These experiments have led to a number of proposed functions for pUL12 that have changed slightly with time. Current theories hypothesize that the protein plays some sort of role in resolving complex, highly branched replication intermediates (18, 20, 29, 31), aids in the formation of capsids (17), or has an undiscovered function (17).

While the majority of the research has been performed with pUL12, these hypotheses apply to other herpesvirus alkaline nucleases as well. This includes the pUL12 homolog found in CMV, the alkaline nuclease pUL98 (18). As mentioned, the herpesvirus alkaline nucleases, while

conserved, have low levels of homology—pUL98 is roughly 22-26% identical and 47% similar to pUL12 (18, 31). However, the active site residues have remained well conserved across the herpesviruses, and any other changes in adjacent domains likely reflect evolutionary pressures and substrate specificity (6, 25). pUL98 is a 65 kDa nuclease with the same reaction conditions and optima as the other herpesvirus alkaline nucleases (25, 31). Using the crystalline structure of KSHV alkaline nuclease, KSHV-SOX, which has been identified as the closest match to pUL98, a predicted model of pUL98 has been created (25). Subsequent analysis revealed two different binding sites, one of which is the active site with an adjacent binding site for the 5' phosphate of the DNA substrate (25). However, the presence of a 'bridge' region in KSHV-SOX has limited the utility of this model as a substitute for the structural model of pUL98, indicating a need for resolution of the crystal structure of pUL98 (25).

Several experiments and findings corroborate the evolutionary relationship and potential analogous roles of HSV-1 pUL12 and CMV pUL98. It is known that like pUL12, pUL98 can localize to the nucleus of host cells independently of other viral proteins, indicating that the protein sequence itself contains the necessary information for transport to and retention within the nucleus (18). Similarly, pUL98 can be detected early after infection, with a drastic increase in concentration following the onset of replication of the viral genome (31). Mutagenesis studies based off of the pUL98 structural model have identified the catalytic residues necessary for both the exo and endonuclease activities of pUL98 (25). Identification and analysis of the effects of mutations in these residues will allow for a greater understanding of the role of pUL98 in CMV infection. Initial experiments investigating the effect of a pUL98 null mutant either inactivated pUL98 via transposon insertion or removed UL98 and replaced it with galK. In both cases, the changes resulted in lethality (25). However, these mutations could have affected adjacent viral

genes, and so an amber nonsense mutation was introduced that maintained the ORF (25). This revealed that, similar to pUL12, pUL98 is not absolutely necessary for viral replication, though the mutant virus was significantly impaired. Although wild type CMV could be detected at a p.f.u. mL^{-1} of 10^4 by 33 days post infection, the amber mutant virus was not detected until 33 days post infection (25). Despite the differences between pUL12 and pUL98, it has been shown that pUL98 can complement the lacZ insertional UL12 HSV-1 mutant (18). These findings indicate that pUL98 and pUL12 may serve similar roles, and that inhibition of pUL98 may result in the ability of the host to clear CMV infection due to attenuated replication of the virus. Additionally, pUL98 represents a different antiviral target that is not affected by current antivirals, and so resistance to currently used compounds is not likely to cause resistance to compounds inhibiting pUL98. Due to the evolutionary history of pUL98, it does not share homology with any known host enzymes, thus suggesting that any active compounds will be unlikely to interfere with host machinery, reducing the possibility of toxic effects of treatment.

New Developments and Project Goals

Several decades ago, the search for new anti-HIV compounds led researchers to discover a number of potential antiviral compounds (4). One group of these compounds, anthraquinones, were found to have inhibitory effects on HSV-1 and 2, EBV, and CMV (4). This led to investigations on the effectiveness of anthraquinone derivatives, such as sulfonated anthraquinones, that had altered properties such as increased solubility (4). In addition to these altered compounds, other researchers looked at various anthraquinones that were isolated from different sources, such as rhubarb (44). Of the anthraquinones derivatives, several were found to be effective against CMV, including acid blue 129, acid blue 40, and alizarin violet R (4).

Another anthraquinone, emodin, was found to be active against HSV, potentially by inhibiting pUL12 (44).

This has led a research group composed of scientists from Virginia Commonwealth University and The Ohio State University, of which I am a member, to examine the effectiveness of the above listed compounds in inhibiting CMV (2). The research not only examined the effectiveness of the compounds in inhibiting viral replication and production in cell culture, but also the toxicity of the compounds to host cells (2). My main role in the recently published paper was to purify active CMV pUL98 and to setup, analyze, and determine the effectiveness of a miniaturized assay that would function in identifying other inhibitors of the CMV alkaline nuclease. In doing this, I examined the effects of acid blue 129 (atanyl blue PRL) and acid blue 40 on the enzyme activity of pUL98 (2). The remainder of this thesis will cover the work that I performed, the conclusions that were reached, and the future directions that this line of research can produce.

Materials and Methods

Gene Construct and Plasmid Construction

See Kuchta et al (25) for information regarding the construction of the plasmid. The double histidine tag and TEV cleavage site were both additions performed by Yali Zhu of the Deborah Parris research laboratory. Additional information regarding their construction can be obtained via correspondence.

Transformation of BL21 (DE3) pLysS

The transformation procedure used was the recommended method provided by Bioline. After thawing on ice, 50 µL aliquots of bacterial cells were mixed via light agitation. To these aliquots, 2 µL of pDest17 UL98 plasmid was added and subsequently mixed and incubated on

ice for 30 minutes. Heat shock (exposure to a 42 °C water bath for 30-45 seconds) was used to induce transformation of the bacterial cells. Following this step, the cells were cooled on ice for 2 minutes.

The transformed bacteria were then diluted to 1 mL by the addition of SOC media (2% tryptone, 0.5% yeast extract, 0.4% glucose, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$). Following dilution, each sample was incubated at 37 °C for 1 hour while shaking at 225 rpm. Various quantities of transformed bacterial cells (10-200 µL) were used to inoculate Luria broth (LB)/ampicillin (1 µg/mL) agar plates. The plates were incubated overnight at 37 °C.

Expression of pUL98

Colonies grown on the LB/ampicillin agar plates were examined, and one colony was selected and inoculated into 50 mL of LB with ampicillin at a final concentration of 1µg/mL. The culture was then grown overnight at 37 °C while shaking at 225 rpm. The next morning, 20 mL of this culture was used to inoculate 1 liter of LB, also with a final ampicillin concentration of 1µg/mL. This culture was incubated at 37 °C while shaking at 225 rpm until the cells reached OD of 0.6-0.8. At this point, the 1 liter culture was induced with 1 mL of 1M IPTG (final concentration 1 mM), and transferred to a room temperature water bath for 5 minutes. The culture was then incubated at room temperature while shaking at 225 rpm for 3 hours.

After induction, the cultures were centrifuged at 4,000 g for 10 minutes at 4 °C. The cell pellets were then washed with 5x protease inhibitors in 5 mL of PBS and re-centrifuged for 10 more minutes at 4,000 g. These washed pellets were then snap frozen in liquid nitrogen and stored in a -80 °C freezer.

A second 1 liter culture was induced with 20 mL of the overnight culture once the first culture was induced. Thus, each expression procedure resulted in the production of 2 liters of cells.

Purification of pUL98

Each pellet from the expression assay was suspended in 10 mL of lysis buffer (20 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole, 10% glycerol, 1% NP-40, 0.015% BME (15 µL/100mL buffer), pH 8.0) containing 5x protease inhibitors and PMSF (5x). Pulse sonication (10-15 seconds over a period of roughly 10-15 minutes on ice) was then used to burst the cells. Samples were centrifuged at 70,000 g for 20 minutes at 4 °C. The supernatant was retained for purification.

Purification of the supernatant occurred over two steps: passage over a Ni-NTA column followed by passage over a Mono Q anion-exchange column. An AKTA fast protein liquid chromatography system using a preset program designed by an earlier member of the Parris lab was utilized. Samples were loaded onto the 5 mL Ni-NTA column equilibrated in lysis buffer and then washed with 40 mL of wash buffer (20 mM Tris-HCl, 300 mM NaCl, 20 mM imidazole, 10% glycerol, 0.015% BME, pH 8.0). Elution was carried out using a linear gradient of 60 ml transitioning from the wash buffer to the elution buffer (20 mM Tris-HCl, 300 mM NaCl, 1M imidazole, 10% glycerol, 1% NP-40, 0.015% BME, pH 8.0), creating an imidazole gradient of 20-500 mM. Those fractions that contained pUL98 by identification using anti-pUL98 I-2 antibody were then pooled and dialyzed overnight in buffer B2 (20 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, 10 % glycerol, 0.015% BME, pH 8.2).

For the Mono Q column, a different program designed by an earlier member of the Parris lab was utilized. The dialyzed Ni-NTA sample was loaded onto the 1 mL Mono Q column and

washed with 10 mL of buffer B2. Elution was carried out using a linear gradient of 25 mL of buffer B2 containing 0-500 mM KCl. To those fractions containing or suspected to contain pUL98 was added 2x protease inhibitors.

Identification of fractions containing pUL98 was done using three different criteria. First, the fractions could be run using SDS-PAGE and stained with Coomassie to visualize the different protein bands. The second method was to perform a Western Blot using the anti-pUL98 I-2 antibody, which specifically marked pUL98 regardless of the presence of other protein. Finally, a radioactivity assay was used to measure the nuclease activity present in each fraction.

Radioactivity Assay

One of two assays used to measure the nuclease activity in the different fractions obtained from purification is the radioactivity release assay. This assay is based on the method used by Hoffman and Cheng (2, 22). In particular, this assay does not discriminate between the exonuclease and endonuclease mechanisms of action. Activity of a sample was measured as the release of trichloroacetic acid-soluble radioactivity from *E. coli* dsDNA uniformly labelled with ^{14}C. In order to standardize results, a unit of activity was defined as the amount of enzyme necessary to digest 1 ng of substrate to solubility in 10 minutes at 37 °C.

Individual reactions were comprised of buffer, enzyme, and substrate, totaling 200 µL in volume. The buffer used consisted of 0.05 M Tris-HCl pH 9.0, 6 mM $MgCl_2$, 1 mM DTT, 0.0162 mg ^{14}C DNA/mL, 0.2 mg/mL BSA, and water. For each reaction, 10 µL of enzyme was assayed (~1100 units). Prior to the addition of enzyme, the reaction buffer was incubated for 5 minutes at 37 °C. Once the enzyme was added, the reactions were carried out for X minutes. The default length of time is 10 minutes, though some time courses were also performed. Water was used in the place of pUL98 for the control reactions. The reactions were stopped via the addition

of 50 µg of unlabeled, sheared salmon sperm DNA and TCA (final concentration to 5%), and subsequently sat on ice for at least 5 minutes. The reaction mixtures were then spun in a tabletop centrifuge at 3000 rpm for 10 minutes. To a scintillation vial was added 7.5 mL of scintillation fluid, 75 µL of 1.2 M NaOH, and 200 µL of the supernatant. The radioactivity was then measured using scintillation spectrometry.

Zen Substrate

A 29-mer oligonucleotide was designed for use in a FRET assay capable of detecting the exonuclease activity of pUL98. The DNA strand forms a hairpin with a 5 bp overhang at the 5' end of the molecule. Bound to the 5' nucleotide is a FAM fluorophore, while an Iowa Black quencher is affixed to the 3' nucleotide. Between nucleotides 9 and 10 is a proprietary compound known as Zen that is used to minimize background fluorescence even more than with the Iowa Black alone (IDT).

The material provided by IDT was diluted 1:10 (final concentration 10 µM) using buffer EB (10mM Tris-HCl, pH 7.5) (Qiagen) and then heated to 95 °C in a water bath for 2 minutes. The substrate was then allowed to cool slowly to a temperature of ~70 °C. After reaching this temperature, the material was quenched on ice and then stored at -20°C. Subsequent use required that the material be thawed in the dark and then distributed to the appropriate reaction vessels.

FRET Assay

The buffer used for the FRET assays was similar to the buffer used in the radioactivity assays, and contained 0.05 M Tris-HCl pH 9.0, 6 mM $MgCl_2$, 1 mM DTT, 0.2 mg/mL BSA, and water. However, they also contained 400 nM Zen substrate as well as 5% DMSO. The potential inhibitors to be tested were dissolved and stored in 100% DMSO and diluted to a final

concentration of 5% prior to their use in inhibition studies. If no inhibitors were being used, 5 µL of 5% DMSO was added instead.

For each FRET assay, the reactions were set up so that each vessel contained all of the components of the buffer, the Zen substrate, and the inhibitor (or control 5% DMSO), totaling 90 µL in volume. The suspensions were then incubated for 5 minutes at 37 °C prior to the addition of 10 µL of pUL98. In place of pUL98, 10 µL of 1 mg/mL BSA was used in the control (no enzyme) reactions. The length of the reaction was altered based on the goals of the experiment. Many of the assays involved time courses, and so samples were run for 10, 30, 45, and 60 minutes, for example. To stop the reaction, EDTA was added to a final concentration of approximately 12 mM. Fluorescence was measured using a SpectraMax M5fluorometer (Molecular Devices), using 384-well plates containing 20 µL aliquots in triplicate from each reaction vessel. Background values obtained from the no enzyme control reactions were subtracted from the experimental readings to obtain relative fluorescence units (RFU).

Inhibition Assays

The FRET inhibition assays were set up and run in the same manner as described above. Instead of using 5 µL of 5% DMSO, 5 µL of inhibitor was added to obtain the final concentration being assayed. Experimental readings were also obtained in the same manner. Activity levels were determined as a percentage of the maximum activity displayed by untreated controls (no inhibitor present). From these data, the 50% inhibitory concentration (IC_{50}) values were obtained using a four-parameter function.

Radioactivity inhibition assays were set up using the same system as presented earlier, only with the addition of DMSO to a final concentration of 5% and the addition of inhibitor to

the desired final concentration. Experimental readings were obtained using the same method as described.

Experiments and Results

Gene Construct

The gene construct used in the following experiments consisted of the wild type UL98 gene spliced to a double Histidine tag at its N terminus (Figure 6). The histidine tag is comprised of two stretches of six histidine residues separated by a short stretch of amino acids. In the linker region between the tag and UL98, there is a tobacco etch virus (TEV) cleavage site. This facilitates the removal of the histidine tag following purification of the protein. In the findings presented here, the tag was not removed, as the nuclease retained significant activity even in the presence of the extra tag domain. The gene construct was then inserted into the Gateway destination vector pDest17 (Invitrogen) for use in transformation and expression procedures. In this system, the gene is under control of a T7 inducible promoter. Further information regarding the construction of the plasmid and gene can be viewed in the Materials and Methods.

A double histidine tag was utilized in order to optimize purification of the pUL98 alkaline nuclease. In the first phase of purification, described later, the bacterial cell lysate was passed over a Ni-NTA column. Histidine has a high affinity for this particular matrix, facilitating the purification of tagged pUL98. The tag is also small enough that it was predicted to not impact the folding of the protein or its activity, which was confirmed in later experiments. Thus, the construct would allow for rapid purification of pUL98, while providing a way to remove the protein tag should it impact enzymatic activity. This method was similar to the one used by Kuchta et al, though a single hexahistidine tag was used in those experiments (25).

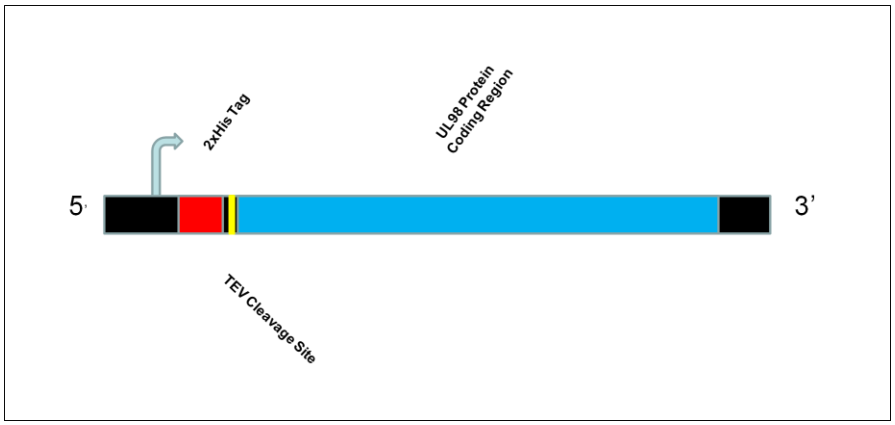

Figure 6 Structure of the UL98 Gene Construct. The protein product of this construct is 623 amino acids in length, and has a molecular weight of 70.1 kilodaltons. The ORF, when in the pDest17 vector, runs from nucleotide 101 to nucleotide 1969. Removal of the histidine tag would have taken place had the protein shown reduced or no functional activity. Additionally, removal of the protein tag would be necessary for any further crystallography studies using this expression system.

Expression of pUL98

The initial expression system was based off of work done by Allison Kuchta and her colleagues (25). BL21 (DE3) pLysS cells were transformed with the expression vector and cultured according to the Materials and Methods. Initially, in order to determine the most efficient conditions for protein production, a culture of *E. coli* cells was grown to an O.D. of 0.6-0.8 and then exposed to IPTG to induce pUL98 expression. The protocol used by Kuchta stated that the culture was to be incubated for three hours after induction. To test this, aliquots were removed every hour until a total of four hours had passed. The bacterial cells in each of these aliquots were lysed and centrifuged, and the protein produced by the cells, both soluble and insoluble, extracted. The extracted protein was then analyzed on denaturing-polyacrylamide gels followed by staining with Coomassie blue (Figure 7).

Figure 7A shows the soluble protein profile of the bacterial cells when uninduced and at several time points following induction. Similar data is presented Figure 7B, though this contains the insoluble protein fraction. Over the course of four hours, the total amount of protein

produced increased with time as expected. By comparing the soluble fraction to the insoluble fraction, it can be seen that the majority of the protein remained soluble.

The pUL98 protein is predicted to have a molecular weight of 70.1 kDa, and so this served as the starting point for identifying the production of this protein. The marked region of the gel in Figure 7A indicates the expected position of a band corresponding to pUL98. Though the upper and lower bands have apparent molecular weights of 103 and 90 kDa respectively, this does not mean that pUL98 is not present. It is possible that the band corresponding to pUL98 cannot be visualized well using Coomassie staining due to low concentrations of pUL98 compared to other proteins in the sample. Additionally, my principle investigator, Dr. Parris, pointed out that in her own research on the HSV-1 DNA polymerase, it is observed that the polymerase migrates at a higher apparent molecular weight than expected due to the composition of the protein. A similar case could be occurring here. Worth noting is the fact that very little protein is revealed in the same region of the gel in Figure 7B, indicating that most of the protein at this approximate size is soluble. Thus, there was evidence showing that the protein of interest could be within the soluble protein fraction, justifying purification.

Using these data, it was decided that all subsequent expression experiments would be performed using a post-induction incubation time of three hours. In comparing the different lanes in Figure 7A, there is more darkly staining material present in the 3 and 4 hour post-induction lanes than in the earlier lanes, indicating the presence of greater amounts of protein. Three hours is preferred over four hours for three reasons. First, as cell density increases and bacteria begin to enter stationary phase, it becomes more difficult to lyse the cells and extract protein. Second, bacterial cells do not have a separate compartment for their genetic information. The protein of interest, being a nuclease, is thus toxic to the bacterial cell. This is the reason that BL21 cells

were utilized, as they keep background expression of genes under control of the T7 promoter low until induced by IPTG. In order to minimize the effect of this toxicity, the bacterial cells should be cultured long enough to obtain adequate amounts of protein and no longer. Finally, as the amount of protein produced by the bacterial cells increases, there is a greater chance of protein crystallization and precipitation.

A comprehensive analysis to determine the optimum conditions for efficient expression of pUL98 was not performed. Such an analysis would be necessary for future work involving production of pUL98 for crystallography studies.

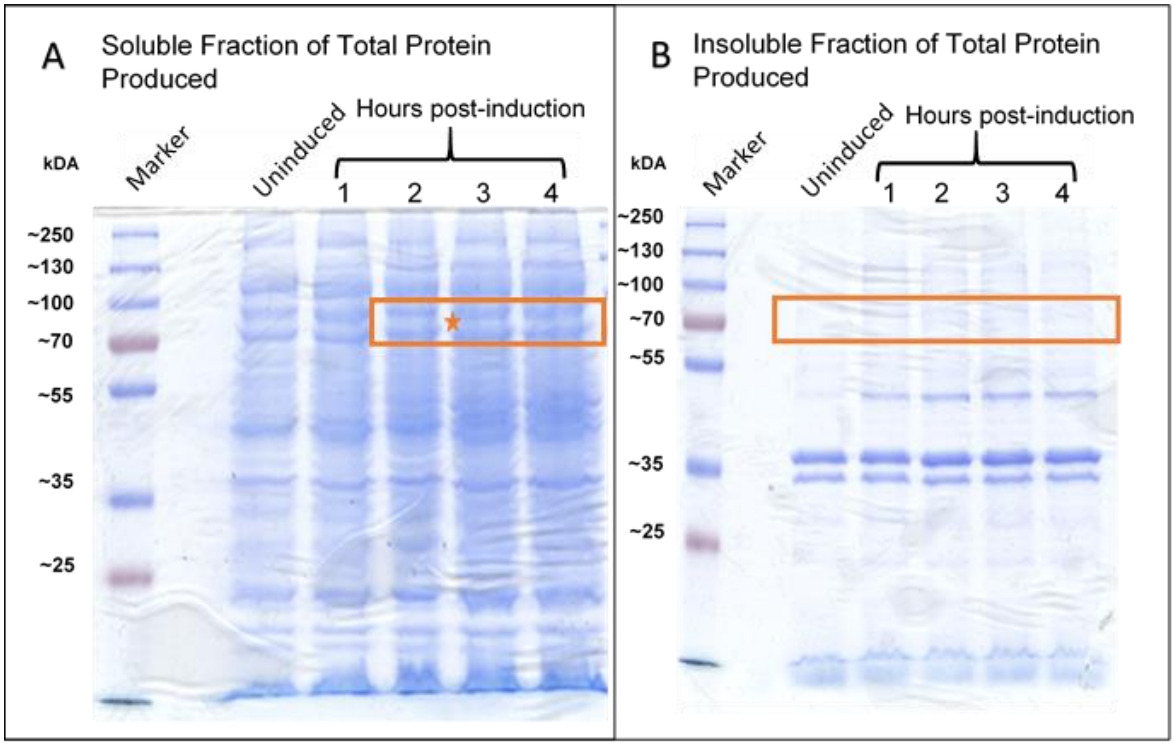

Figure 7 Coomassie Staining of Protein Extracted from *E. coli* Cultures. A) The upper and lower protein bands within the box have apparent molecular weights of 103 kDa and 90 kDa. The star indicates that 3 hours post-induction represents the time at which all subsequent purifications were performed. B) There is very little protein that is showing within the boxed region, especially when compared to the darkly staining low bands at ~35 kDa.

Purification of pUL98

Cultures were centrifuged, snap frozen in liquid nitrogen, and stored in a -80°C freezer for up to several months until purification procedures were executed. Frozen aliquots were suspended in lysis buffer, subjected to ultrasonic disruption, and the insoluble material removed by centrifugation. Sequential Ni-chelating and a Mono Q anion exchange columns as described in the Materials and Methods were used to purify pUL98.

In order to obtain purified pUL98, it was necessary to determine which elution fractions contained the protein. During passage of the sample over the Ni-chelating column, the elution of protein could be observed via a change in the optical density of the eluent. This information directed our attention to specific fractions being collected from the column. Initially, the presence or absence of pUL98 was determined via Coomassie staining and Western Blotting (Figure 8).

Those fractions that contained protein, as determined by their optical density, had to be analyzed to determine whether or not the eluted material included pUL98. Coomassie staining of the relevant fractions, as well as several preceding and succeeding fractions, was utilized to probe for the presence of pUL98 (Figure 8A). The boxed region in Figure 8A contains two different protein bands at apparent molecular weights of 88 and 78 kDa. Included in this data are the Ni-chelating column input and the collected flow-through (unbound), which appear almost identical (as is expected for a column with affinity for His tags specifically). Little to no protein can be visualized in the fractions flanking the lanes corresponding to fractions 31, 32, and 33.

The predicted size of pUL98 and whether or not there were protein bands corresponding to this size was used as criteria for determining whether or not the fraction contained pUL98 in early purifications. Protein bands such as those in Figure 8A were considered to be indicative of containing pUL98. Though the bands do not match the exact size of pUL98, they are close

enough in apparent size that further purification and analysis of those fractions was warranted. Furthermore, these fractions corresponded with the change in optical density observed during elution of the sample from the column (data not included). Samples that were suspected to contain pUL98 were pooled, dialyzed, and subsequently passed over the Mono Q anion exchange column (see Materials and Methods).

Protein elution from the Mono Q column could be observed through a change in the optical density of the fraction, as was the case with the Ni-chelating column. This again directed attention to particular fractions that were then analyzed by both Coomassie staining and Western blotting. Figures 8B and 8C present two different Western blots, each of which utilized a different antibody. In Figure 8B, an antibody that specifically recognized the His tag was utilized while an antibody specific to pUL98 was used in Figure 8C. While each lane containing a sample from a Mono Q fraction contains a signal in Figure 8B, so too does the lane containing the molecular ladder. In contrast, in Figure 8C, the lane containing the molecular ladder does not display any signal. However, the antibody to pUL98 also shows signals in each of the lanes corresponding to a different Mono Q fraction. Included in these data is a Mono Q input sample, which displays several signals at varying molecular weights.

It was initially planned that only the commercially available His antibody would be used to perform Western blots and to track the His-tagged pUL98. However, it was immediately clear that the antibody had poor specificity. The marker lane should not contain any of the antibody signal as none of the included proteins contain a His tag, yet that is exactly what is observed in Figure 8B. Since conclusive evidence demonstrated this nonspecificity, any other signals observed in blots using the His antibody could not be considered signals marking the presence of pUL98. This led to the use of the pUL98 antibody, which demonstrated a much better specificity

and allowed for clear determination of fractions containing pUL98 (Figure 8C). The presence of several bands in the Mono Q input lane most likely indicates the presence of several truncated variants of pUL98. Since pUL98, as identified by antibody specific to the protein, could be obtained using this procedure, this demonstrates that the protein bands in Figure 8A act as markers for the presence of pUL98. Though they do not have the correct apparent molecular weight, they are close in size and yield pUL98 when further purified by passage over the Mono Q column.

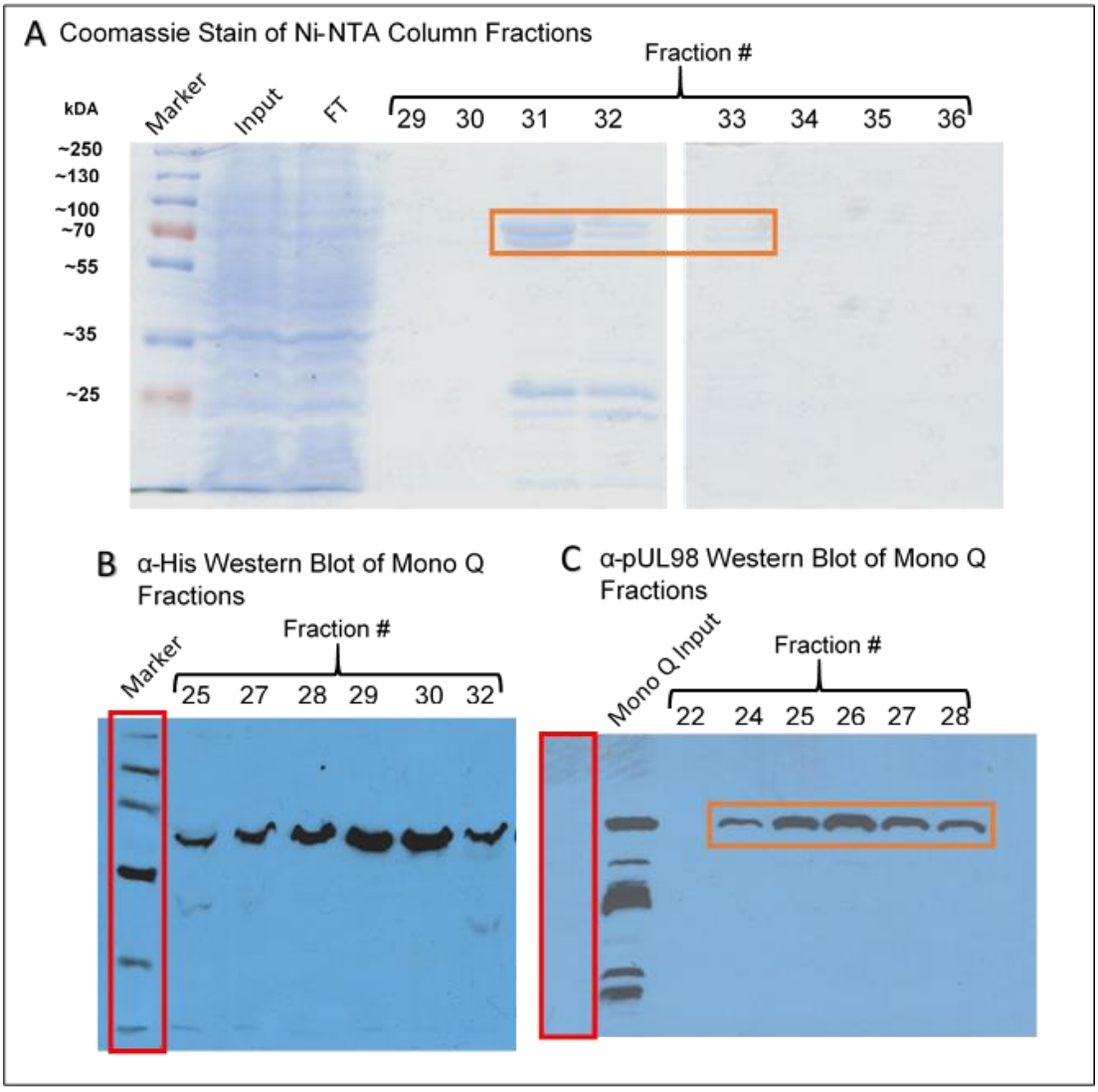

Figure 8 Tracking the Elution of Protein Using Coomassie Staining and Western Blots. A) The boxed region contains 2 protein bands at 88 and 78 kDa. These bands were initially used as markers for putative pUL98 containing fractions. B) The red box indicates that the marker lane was being visualized despite the absence of His tags. The His antibody is not specific under the conditions used. C) The red box shows that the marker bands are not visualized when using the α-pUL98 antibody. The orange box contains signals indicating the presence of pUL98.

While Coomassie staining and Western blots had been used to track and identify those fractions containing pUL98 in early purifications, they could not determine whether or not the protein was active. Thus, a different assay was needed to test whether or not the pUL98 that was obtained still retained its nuclease function. The activity of various fractions was analyzed using the radioactivity release assay as described in the Materials and Methods. Initially, such an assay

was utilized to confirm which fractions contained active pUL98. However, the assay came to be used as another method of tracking the purification of pUL98 as it was sequentially passed over the Ni-chelating and Mono Q columns. Since the presumed relevant function of pUL98 is its nuclease activity, it was deemed more efficient to track the protein over the course of its purification using an assay that specifically indicates whether or not a fraction contains nuclease activity. Those fractions that contained the most activity could then be further analyzed via Western blots. The radioactivity assay, in combination with the pUL98-specific antibody Western blots, were key in deciding which fractions contained the protein of interest for both purification purposes and use in later experiments.

Figure 9 demonstrates the utility of the radioactivity release assay, both in identifying those fractions containing pUL98 activity as well as quantifying that activity. A representative selection of Mono Q fractions was assayed in Figure 9A, revealing the presence of a peak of maximal activity. There is a sharp increase in the activity of the fractions as the peak begins that trails in the later fractions. Identification of the relevant fractions narrowed down the range over which it was necessary to test for the presence of pUL98 by Western blot (Figure 9B). In addition to signals corresponding to pUL98, Figure 9B also contains signals that again indicate the presence of several truncated variants of pUL98.

Several key pieces of information were obtained by using and comparing radioactivity release assays and Western blots. First, nuclease activity corresponds directly with the presence of pUL98, and is not due to a contaminating factor. Such comparisons also validated the combined use of these two different methods to identify pUL98. Finally, it demonstrates that the presence of pUL98 as identified via Western blot does not necessarily mean that the fraction contains active pUL98. Thus, the use of the radioactivity release assay is necessary to identify

the fractions of interest. Such data can be observed by comparing Figure 9B to 9A. While fraction 25 contains both significant activity and immune-reactive pUL98 signal, fraction 22, which contains pUL98 signal, has less than half of the activity of the three most active fractions (23, 25, and 27). This demonstrates that the Western blot cannot be used alone to identify fractions of interest. It also demonstrates that there are fractions that contain what is thought to be full length pUL98 but have significantly lower activity than expected. This would result from inactivation of pUL98 so that it can no longer function appropriately, though the reason(s) for such a situation were not explored.

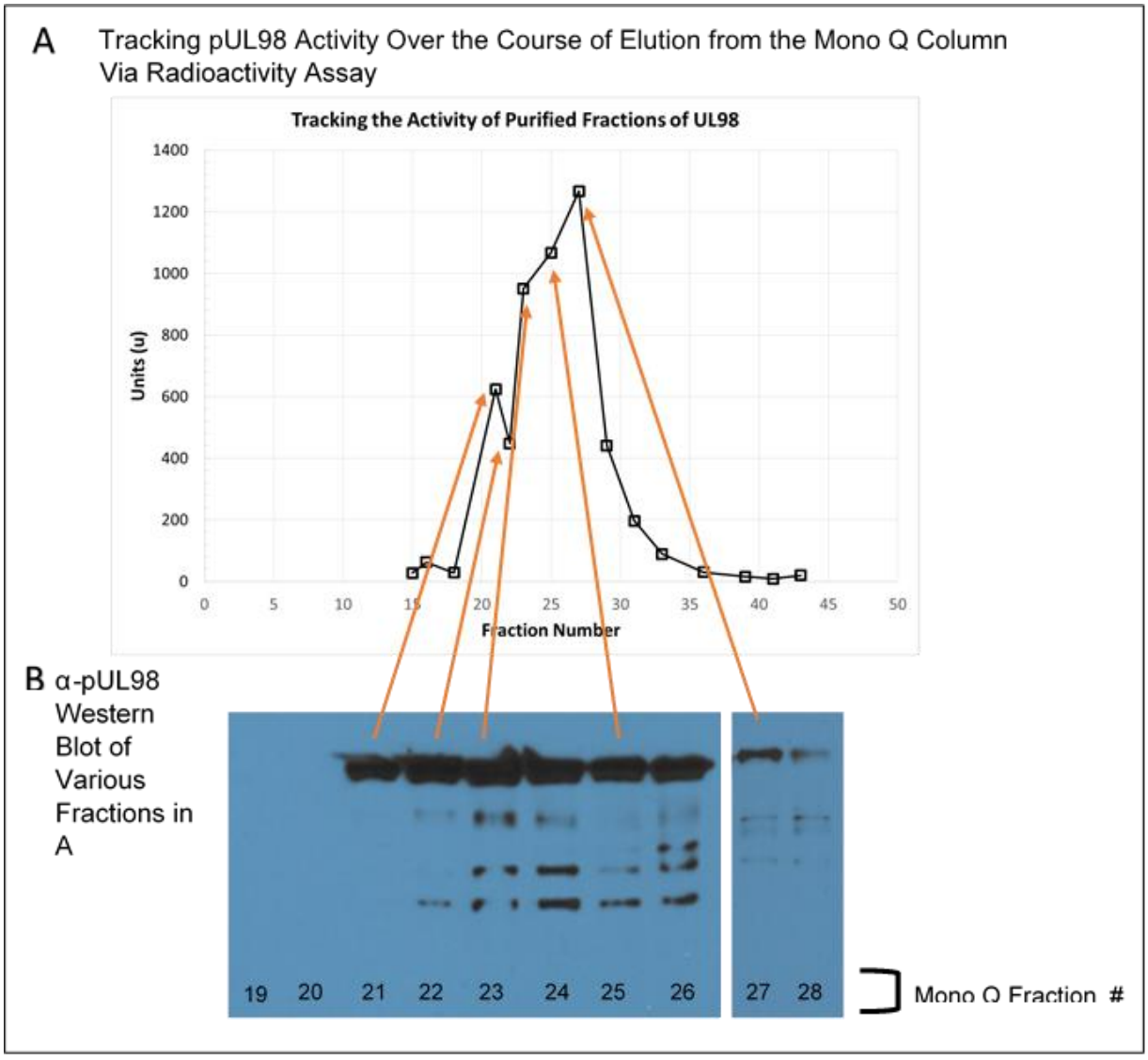

Figure 9 pUL98 Activity Compared to Mono Q Western Blots. Analyzing the elution fractions for the presence of pUL98 activity indicates that those fractions with nuclease activity contain protein detectable by the α-pUL98 antibody.

FRET Assay Design and Testing

While the radioactivity assay is effective in quantifying the activity of pUL98, it is not ideal for several reasons. It requires the use of radioactive substrate, which requires significant investments of time and resources to prepare and maintain. Such material also requires greater care and oversight when being used. Compounding this problem is the fact that relatively large amounts of substrate are necessary in the release assays. Measurement and quantification of the

results of such assays also requires significant investments of time, as a scintillation counting machine takes significantly more time to measure a sample than does a fluorimeter or a spectrometer. The relatively large volume of each reaction prohibits high throughput screening as well.

An alternative to the use of radioactive substrate in a release assay is the use of fluorescence resonance energy transfer (FRET) to quantify nuclease activity. FRET assays rely on the design of the substrate to work appropriately. When struck by light of the appropriate wavelength, a fluorophore absorbs the energy of the light and then emits light at a longer wavelength (lower energy) as excited electrons fall back to ground state. Chemical entities known as quenchers are capable of absorbing this emitted light when the quencher is in close proximity to the fluorophore. This relationship between fluorophore and quencher is critical in the use of a FRET assay. The substrate is designed in such a way that the quencher is kept nearby the fluorophore, preventing the emission of light. When the substrate is exposed to the protein of interest, in this case a nuclease, the fluorophore is cleaved and no longer in close proximity to the quencher. This allows the release of fluorescence that can then be detected via fluorimeter as a measurement of the activity of the nuclease.

The design of the FRET assay used in my experiments is based off of prior work done by Allison Kuchta, a prior M.D./Ph.D. student from VCU who worked in the Parris lab. However, it was determined that the fluorescently labeled DNA substrate used in her assays was not ideal for my purposes for several reasons. In terms of design, the previous substrate relied on proximity and chemical affinity between the fluorophore and its quencher. This resulted in high levels of background fluorescence in the absence of any nuclease activity (data not shown). High background readings made it difficult to quantify the activity of pUL98, as the fluorescence

release quickly went beyond the linear range of the fluorimeter. Additionally, the design of the substrate was such that both exo and endonuclease activity could result in the detection of fluorescence. The main function of pUL98 is believed to be its exonuclease activity, due to significantly higher levels of this type of activity over endonuclease activity observed in previous experiments (31). The exonuclease activity of the pUL98 homolog, pUL12, has also been shown to be necessary for wild type viral production, as noted earlier (20, 22). Therefore, the exonuclease activity of pUL98 is the key function being analyzed in my experiments. Thus, the specificity of the substrate and what it measured was less than ideal, a problem which was exacerbated by the high levels of background fluorescence.

I designed a new substrate for use in the following FRET assays that not only had significantly lower background, but also favored quantification of the exonuclease activity of pUL98 specifically (Figure 11). Figure 11A shows the design of the substrate, including both the sequence of the oligonucleotide as well as the locations of the various components specific to a FRET assay. Unlike the previous substrate, this one relies upon intramolecular base pairing to form a hairpin with a 5' overhang that positions the fluorophore (FAM) in close proximity to the quencher (Iowa Black). Additionally, a proprietary compound known as Zen was utilized to further decrease the background fluorescence of the substrate (IDT). The formation of a hairpin also allows the substrate to favor exonuclease activity over endonuclease activity, as indicated by the mechanism of fluorescence release in Figure 11B. Though endonucleolytic cleavage can still occur, it is unlikely to cause the release of the fluorophore due to the base pairing present. Several endonucleolytic events would be needed to cause release of significant amounts of fluorescence. However, the FRET assays used were timed, thus allowing observation of the linear range of fluorescence release, which is consistent with exonuclease activity. Furthermore,

since the fluorophore is directly linked to the 5' nucleotide, any exonuclease activity will result in the release of the fluorophore from the substrate, allowing detection of fluorescence.

The conditions necessary for the FRET assay had to be determined over the course of several different experiments (data not shown). The correct concentration of substrate needed to be determined so that the fluorescence detected remained within the sensitive range of the instrument. At the same time, enough substrate needed to be present to be able to accurately quantify the activity of pUL98. Time was another important factor, as the activity of pUL98 needed to remain within the linear range of fluorescence release. Such conditions were elucidated by performing several variants of the FRET procedure in the Materials and Methods, where the effect of different variables was examined (different times, various substrate concentrations, etc.). This resulted in the FRET assay utilized in the following experiments and data.

Figure 10 demonstrates the complete process from purification to FRET assay, as well as validates the proof-of-concept of the assay itself. Figures 10A and 10B show the Coomassie stain and corresponding Western blot for several of the Mono Q fractions. Those bands in the Coomassie stain that act as markers for the presence of pUL98 (as determined earlier) have apparent molecular weights between 96 and 103 kDa. The Western blot confirms the presence of pUL98 within these fractions, which were then assayed for nuclease activity using the radioactivity release assay (Figure 10C). A peak of activity, corresponding to fractions 25 and 26, is observed and serves as the basis for using these fractions in the FRET assay in Figure 10D. Each fraction was assayed separately using the FRET assay, and a DNase I control was included. The activities of the Mono Q fractions were very similar, and significantly different from the

activity of the endonuclease DNase I. It appears that the linear range of fluorescence release for pUL98 occurs from 0 to 120 minutes at the concentrations tested.

Significant activity of fractions 25 and 26 in both the FRET assay and the radioactivity release assay demonstrates the ability of these different assays to quantify nuclease activity—nuclease activity in the radioactivity release assay corresponded to nuclease activity as determined via FRET assay. As expected, the pUL98-containing fractions displayed significantly greater activity in the FRET assay than did DNase I. Thus, the specificity of the timed assay for detecting exonuclease activity was validated. The elucidation of the linear range of fluorescence release was critical, as this information was necessary for later inhibition experiments. All inhibition experiments, as described in the Materials and Methods, were performed for no more than 75 minutes, and most were performed to a maximum of 60 minutes, well within the linear range of fluorescence release.

The differences between the FRET assay and the radioactivity release assay demonstrate the favorability of the FRET assay. Significantly lower amounts of DNA substrate per amount of fraction material are required for the FRET assay while retaining the ability to effectively quantify pUL98 activity. Smaller reaction volumes allow for the screening of nuclease activity using 384 well plates, thus enabling a greater ability to assay pUL98 activity under different conditions and from different sources (fractions) in a lesser amount of time. A generally lesser amount of all reaction components means lower costs are required for the FRET assay. Such characteristics—lower cost, time, and materials combined with increased coverage—are needed for designing a high throughput assay to test inhibitors for their ability to inhibit the activity of pUL98.

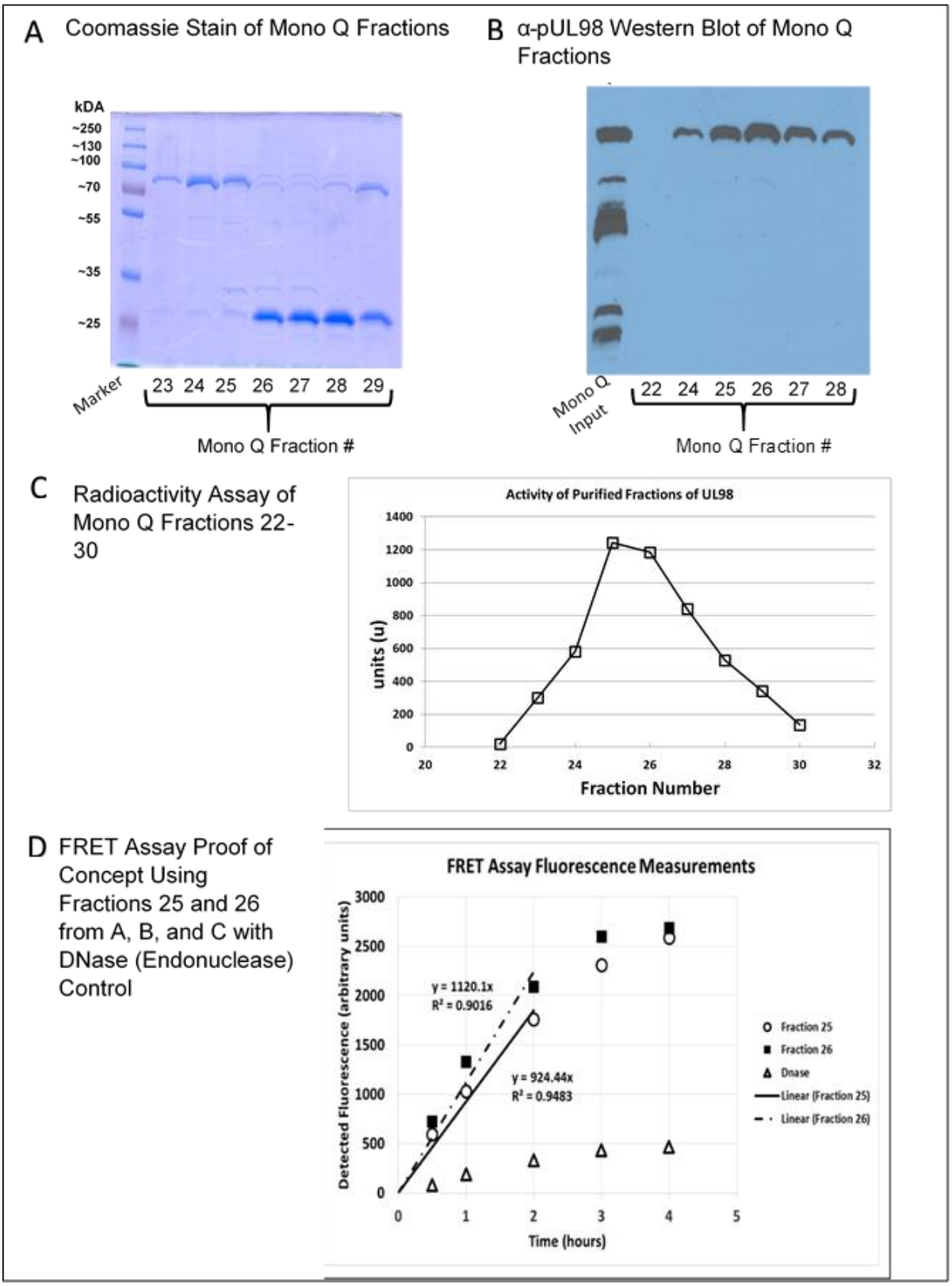

Figure 10 Purification of pUL98 and Proof of Concept of the FRET Assay. A) The bands appearing between markers 100 kDa and 70kDa have apparent molecular weights between 96 and 103 kDa. B) The presence of the bands in A) was used as a

marker for putative pUL98 containing fractions, as confirmed via α-pUL98 Western Blotting. C) Activity profile of the fractions identified in A) and B) demonstrating a peak of nuclease activity. D) Fractions 25 and 26 were determined to contain the greatest amount of active pUL98. These fractions were then used to demonstrate the validity and effectiveness of the FRET assay.

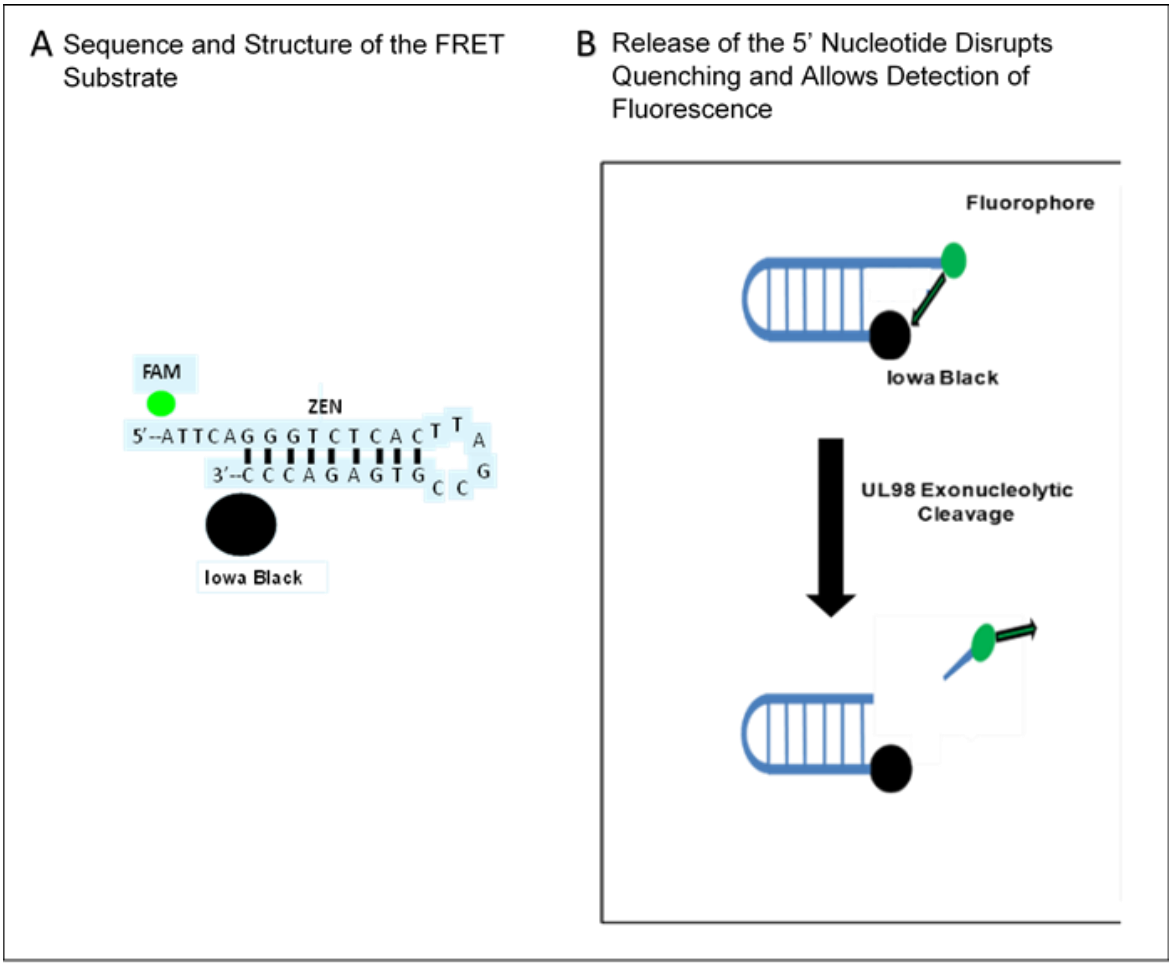

Figure 11 FRET Assay Substrate and Detection of Activity.

Inhibition Assays

With the proof-of-concept of the FRET assay complete, it was time to move onto the final phase of the process—the testing of different compounds for their effectiveness as inhibitors of pUL98. Through the use of *in silico* modeling and testing, collaborators at VCU were able to identify a set of compounds capable of interacting with the putative active site of pUL98. A subset of these compounds was sent to the Parris lab for testing and used in the newly

designed FRET assay. Through *in vitro* assays performed by VCU, it was determined that two compounds would be the focus of the FRET inhibition assays—Acid Blue 40 and Acid Blue 129 (atanyl blue PRL). It was determined by VCU that Acid Blue 129 was more effective at inhibiting CMV than was Acid Blue 40, due to its lower EC_{50} (2). Acid Blue 129 was shown by our collaborators to inhibit CMV replication in cell culture, and that inhibition appears to occur at an early stage in the replication cycle (2). Though Acid Blue 40 had inhibitory effects on CMV, high concentrations that were similar to the cytotoxicity of the compound were required (2). These compounds, Acid Blues 40 and 129, are anthraquinone derivatives. As noted earlier, anthraquinone derivatives have been shown to have inhibitory effects against CMV in other studies. Since anthraquinone derivatives have activity against CMV, Acid Blue 129 appears to inhibit an early step in the replication cycle of CMV, pUL98 can be detected at early times post infection, and Acid Blue 129 has been proposed via *in silico* modelling to interact with the active site of pUL98, it was hypothesized that inhibition of pUL98 may be the mechanism by which inhibition of CMV replication in cell culture occurs. Thus, the results of FRET assay inhibition experiments could provide evidence of a) whether or not these compounds are inhibiting pUL98, b) the validity of the FRET assay, and c) the possibility of an early function for pUL98 in the viral life cycle.

Figure 12A shows data obtained from early inhibition experiments using the radioactivity release assay. The data indicates that pUL98 activity is inhibited by the presence of these putative inhibitory agents in the reaction mixture. In agreement with the findings of VCU, Acid Blue 129 was found to have a greater inhibitory effect than Acid Blue 40—the IC_{50} of Acid Blue 129 was determined to be two orders of magnitude lower than the IC_{50} of Acid Blue 40 (6 vs 100

μM respectively). Figure 12B presents the same data and information that is in 12A, only in a different format.

These data indicated that further testing of the inhibitory compounds against pUL98 using the FRET assay were both warranted and promising. It does appear that pUL98 is inhibited directly by these compounds, and thus provides evidence that this may be the mechanism of inhibition of CMV replication. As expected from the results provided by VCU, Acid Blue 129 was more active against pUL98 than was Acid Blue 40. The presentation of the data in Figure 12B allows for comparisons between inhibition studies using the radioactivity release assay and the FRET assay (Figure 12C).

Rather than compare the fluorescence detected for uninhibited pUL98 to that obtained using each concentration of inhibitor, it was decided that the rates of fluorescence release would be compared in the final assay. The variability in readings between independent replicates could potentially skew results. Thus, rather than look at absolute values of detected fluorescence, it is more accurate to look at the average rates of release as a metric of pUL98 activity. In order to do this, a time course was performed for each concentration of inhibitor, with time points taken at 10, 30, and 45 minutes (well within the linear range determined in Figure 10D). From these data, a rate of fluorescence release by pUL98 activity was determined for each concentration and then compared to the maximum release rate (uninhibited pUL98).

The data from these FRET inhibition experiments is presented in Figure 12C. The format of these data is the same as the format of the radioactivity release data in Figure 12B. From these data, the IC_{50} of Acid Blue 129 is 9.3 μM while the IC_{50} of Acid Blue 40 is 149 μM. Both curves are representative of a standard dose response to the presence of increasing concentrations of inhibitor.

As with the radioactivity assay and the preliminary results from VCU, Acid Blue 129 was found to have a greater inhibitory effect than Acid Blue 40. The IC_{50} values for both of these compounds were similar between the radioactivity release assay and the FRET assay—6 and 9.3 μM for Acid Blue 129 and 100 and 149 μM for Acid Blue 40, respectively. Thus, the FRET assay is useful as both a method of screening for pUL98 activity as well as a platform with which to test the inhibitory effects of different compounds.

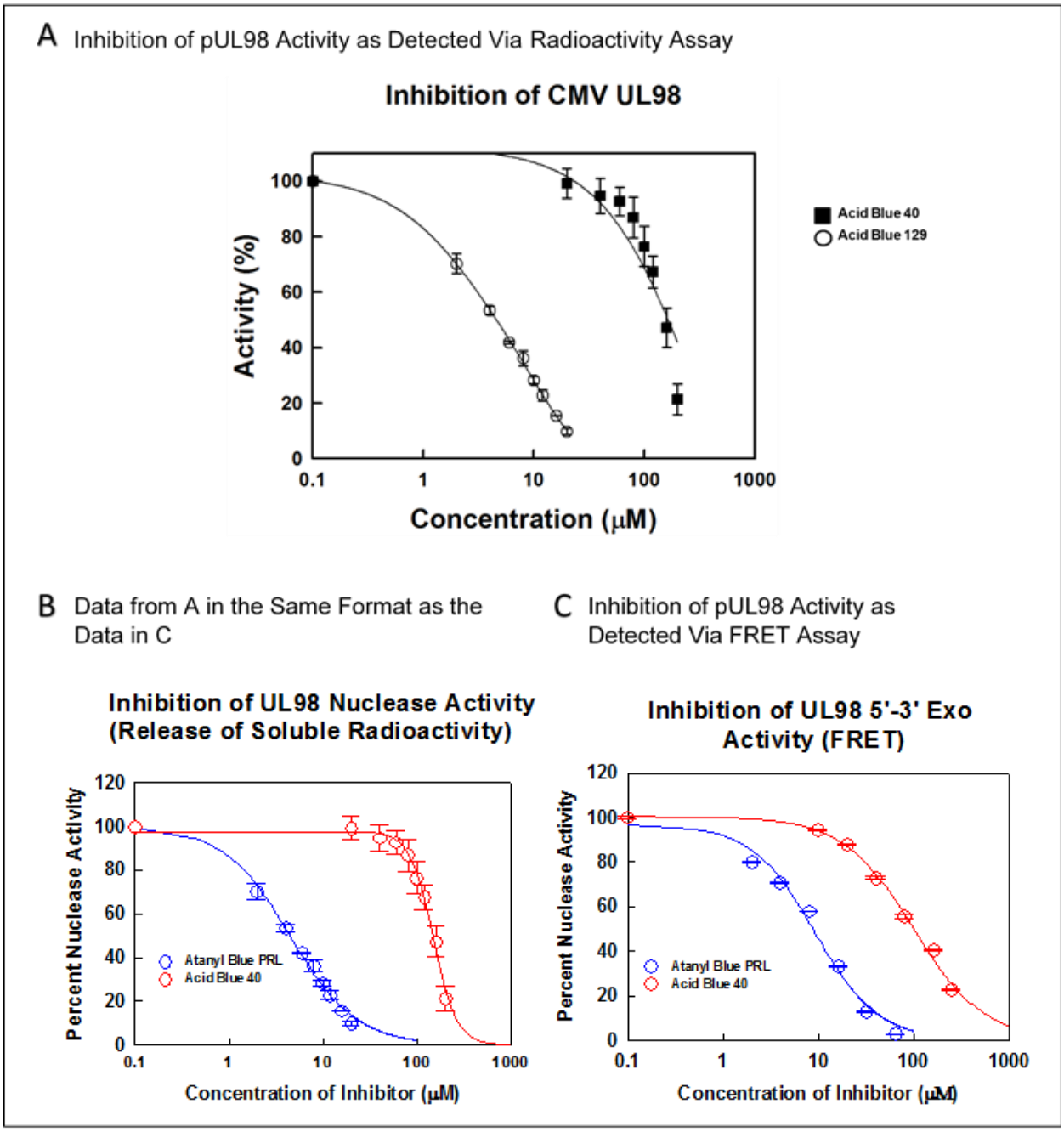

Figure 12 Inhibition of pUL98 Activity. A) Effects of various concentrations of each inhibitor on the activity of pUL98 as quantified using the radioactivity assay. Acid Blue 129 has a much lower IC_{50} (~6 μM) than does Acid Blue 40 (~ 100 μM). These results indicated that the compounds likely inhibit pUL98, and that further testing using the FRET assay was promising. B) The same data as presented in A), only this time matched to the same curve and layout as the FRET assay in C). C) Inhibition of pUL98 activity as quantified by the FRET assay. The IC_{50} for Acid Blue 129 is 9.3 μM while the IC_{50} for Acid Blue 40 is 149 μM. B and C are adapted from Figure 6 in the Alam et al paper (2).

Discussion and Future Work

CMV is a ubiquitous viral pathogen capable of causing severe complications in infected individuals, particularly those who are immunocompromised. Current antiviral treatments are limited in nature due to their toxicities, side effects, and the emergence of drug resistant CMV strains. Thus, there is a need for new antiviral compounds that target viral machinery other than the viral DNA polymerase. This was the motivation behind the work presented here. Previous research on one of the proteins utilized by CMV, the alkaline nuclease pUL98, indicated that it may serve to be a promising target for antiviral therapy. The use of *in silico* modeling allowed a subset of potential inhibitory compounds to be isolated that may be active against pUL98. These compounds were subsequently tested for their effectiveness as inhibitors of the nuclease using two different assays. The FRET assay was designed in tandem with the testing of the compounds to create and optimize a system for high throughput testing of these compounds.

Two compounds, Acid Blues 129 and 40, were discovered to have inhibitory action against CMV at a step early in the viral life cycle, following entry of the virion into the host cell (2). One potential mechanism of inhibition at such a stage would be the inhibition of the activity of pUL98. This hypothesis was tested using purified nuclease in both radioactivity release and FRET assays. These assays indicated that the compounds did have inhibitory effects on pUL98, and that the concentrations observed to be effective at inhibiting UL98 enzyme activity were similar to those observed to inhibit the replication of CMV in cell culture (2).

One alternate possibility for the mechanism of inhibition of Acid Blues 129 and 40 is that they are intercalating into the substrate DNA due to their chemical nature, thus disrupting pUL98 activity by disrupting its substrate. Such a mechanism would be devastating to a host if these compounds were to be used as antivirals. It is worth noting that the negative impact of intercalating agents on host cells may not be immediately apparent—such compounds can be

lethal, but they are also mutagenic, and could cause later effects. However, the data thus far do not support this possibility. In both types of assays, there is a typical inhibitory dose-response observed—as the concentration of inhibitor increases, the activity of pUL98 decreases in a typical dose-response fashion. If intercalation was the cause of this decrease, one would not expect to see this type of response to different concentrations of inhibitors. This is because a typical dose-response curve depends on the kinetics of the enzyme and interaction with the active site of said enzyme. With intercalation, there is no direct interaction between the pUL98 active site and the inhibitor—rather, the inhibition of activity would result from the inability of pUL98 to interact with its substrate appropriately. The IC_{50} values for Acid Blue 129 were very similar when comparing the FRET inhibition assay to the radioactivity release inhibition assay—9.3 µM and 6 µM respectively. However, there was a much greater amount of DNA substrate present in the radioactivity release assay than in the FRET assay. Thus, if the inhibitor was functioning through intercalation, it would be expected that the IC_{50} for the radioactivity assay would be much higher than the value for the FRET assay, as a greater amount of inhibitor is needed to intercalate into the greater amount of DNA present.

The main function of pUL98 in viral replication may be its ability to act as an exonuclease, as indicated by previous research (31). The FRET assay developed was shown to be effective at not only characterizing the activity of pUL98, but at characterizing primarily the exonuclease activity of the protein. Any inhibition seen also indicated that the compound was preventing pUL98 from acting in this manner. Therefore, these results provide evidence that inhibition of the exonuclease activity of pUL98 is correlated with the inhibition of CMV seen in *in vitro* studies.

Finally, the data provide clear proof-of-concept for the use of the FRET assay in studying the inhibitory effects of large numbers of potential antiviral compounds. Though only two compounds were tested, the design of the system allows for many replicates of many different inhibitors at many concentrations to be assayed. A high-throughput FRET assay would also use extremely small quantities of purified enzyme and DNA substrate and could be automated. This can significantly increase the efficiency and speed with which potential antiviral compounds are identified and characterized. It is also possible that the assay could be altered to allow for the characterization of other nucleases, and the discovery of inhibitors for those pathogens utilizing them.

There are several avenues that have opened up as a result of this work. The optimal conditions for expression of pUL98 were not explicitly determined. Though the amount of protein obtained was sufficient for these experiments, an optimized method could result in significantly higher levels of protein. Additionally, such methods could be used at a larger scale to obtain enough protein for crystallography studies. No characterization was performed for pUL98 with the 2xHis tag cleaved from the N terminal end of the protein. Though cleavage of the tag was not necessary for the enzyme inhibition studies performed herein, it is not clear whether the tagged variant has significantly altered activity. Moreover, it would be important for starting material for crystallography studies to have the tag removed. In general, the 2xHis tag was not as useful as anticipated. Though it allowed for a first step purification using a Ni chelating column, it was not effective as a tag to be recognized by antibodies in Western blots. While I-2 α-pUL98 antibody was excellent for tracking the protein, its limited availability would preclude its routine use in pUL98 purification. There may exist a better way to tag and track the protein that does not rely on such a specialized antibody.

There is more work to be done with respect to the inhibition of pUL98 and how it relates, if at all, to the inhibition of CMV in *in vitro* studies. In order to determine whether or not inhibition of pUL98 is the mechanism by which the virus is inhibited, mutant variants of virus resistant to Acid Blue 129 will need to be identified and isolated. The genomes of such mutants can then be sequenced, to see if any mutations conferring resistance correspond to changes in the gene for pUL98. If so, these mutant variants of pUL98 can be isolated and characterized for activity in the presence of inhibitor using the FRET assay. This is just one avenue that makes use of the assay designed in this research.

Works Cited

1. Ahmed, A. (2011). Antiviral Treatment of Cytomegalovirus Infection. Infectious Disorders-Drug Targets, 11(5), 475-503.
2. Alam, Z., Al-Mahdi, Z., Zhu, Y., McKee, Z., Parris, D. S., Parikh, H. I., McVoy, M. A. (2015). Anti-cytomegalovirus activity of the anthraquinone atanyl blue PRL. Antiviral Res, 114, 86-95. doi: 10.1016/j.antiviral.2014.12.003
3. Albà Mm, D. R. O. C. A. K. P. (2001). Genomewide function conservation and phylogeny in the Herpesviridae. Genome research, 11(1), 43-54.
4. Barnard, D. L., Fairbairn, D. W., O'Neill, K. L., Gage, T. L., & Sidwell, R. W. (1995). Anti-human cytomegalovirus activity and toxicity of sulfonated anthraquinones and anthraquinone derivatives. Antiviral Research, 28(4), 317-329.
5. Bazanów, B. A. J. N. A. F. A. B. S. Z. M. (2014). Abortogenic viruses in horses. EVE Equine Veterinary Education, 26(1), 48-55.
6. Bujnicki, J. M., & Rychlewski, L. (2001). The herpesvirus alkaline exonuclease belongs to the restriction endonuclease PD-(D/E)XK superfamily: insight from molecular modeling and phylogenetic analysis. Virus Genes, 22(2), 219-230.
7. Burny, W., Donner, C., Liesnard, C., & Marchant, A. (2004). Epidemiology, pathogenesis and prevention of congenital cytomegalovirus infection. Expert Review of Anti-infective Therapy, 2, 881+.
8. Cardone, G., Heymann, J. B., Cheng, N., Trus, B. L., & Steven, A. C. (2012). Procapsid assembly, maturation, nuclear exit: dynamic steps in the production of infectious herpesvirions. Adv Exp Med Biol, 726, 423-439. doi: 10.1007/978-1-4614-0980-9_19

9. Chayavichitsilp, P., Buckwalter, J. V., Krakowski, A. C., & Friedlander, S. F. (2009). Herpes Simplex. Pediatrics in Review, 30(4), 119-130. doi: 10.1542/pir.30-4-119
10. Davison, A. J. (2010). Herpesvirus systematics. Veterinary microbiology, 143(1), 52-69.
11. Davison, A. J. (2011). Evolution of sexually transmitted and sexually transmissible human herpesviruses. Annals of the New York Academy of Sciences, 1230, 37-49.
12. Davison Aj, E. R. E. B. H. G. S. M. D. J. M. A. C. P. P. E. R. B. S. M. J. T. E. (2009). The order Herpesvirales. Archives of virology, 154(1), 171-177.
13. Di Cocco, P., Soker, T., Clemente, K., Margiotta, G., Coletti, G., Lombardi, L., Pisani, F. (2012). Cytomegalovirus and gastric cancer after renal transplantation: a possible interplay. Transplant Proc, 44(7), 1912-1915. doi: 10.1016/j.transproceed.2012.06.051
14. Field, H. J., & Biswas, S. (2011). Antiviral drug resistance and helicase-primase inhibitors of herpes simplex virus. Drug Resistance Updates: Reviews And Commentaries In Antimicrobial And Anticancer Chemotherapy, 14(1), 45-51. doi: 10.1016/j.drup.2010.11.002
15. Field, H. J., & Vere Hodge, R. A. (2013). Recent developments in anti-herpesvirus drugs. British Medical Bulletin, 106, 213-249. doi: 10.1093/bmb/ldt011
16. Fishman, J. A. (2013). Overview: cytomegalovirus and the herpesviruses in transplantation. American Journal Of Transplantation: Official Journal Of The American Society Of Transplantation And The American Society Of Transplant Surgeons, 13 Suppl 3, 1-8. doi: 10.1111/ajt.12002

17. Fujii, H., Mugitani, M., Koyanagi, N., Liu, Z., Tsuda, S., Arii, J., Kawaguchi, Y. (2014). Role of the nuclease activities encoded by herpes simplex virus 1 UL12 in viral replication and neurovirulence. Journal Of Virology, 88(4), 2359-2364. doi: 10.1128/JVI.03621-13
18. Gao, M., Robertson, B. J., McCann, P. J., O'Boyle, D. R., Weller, S. K., Newcomb, W. W., Weinheimer, S. P. (1998). Functional conservations of the alkaline nuclease of herpes simplex type 1 and human cytomegalovirus. Virology, 249(2), 460-470.
19. Gilbert, C., & Boivin, G. (2005). Human cytomegalovirus resistance to antiviral drugs. Antimicrobial Agents And Chemotherapy, 49(3), 873-883.
20. Goldstein, J. N., & Weller, S. K. (1998). The exonuclease activity of HSV-1 UL12 is required for in vivo function. Virology, 244(2), 442-457.
21. Heming, J. D., Huffman, J. B., Jones, L. M., & Homa, F. L. (2014). Isolation and characterization of the herpes simplex virus 1 terminase complex. Journal Of Virology, 88(1), 225-236. doi: 10.1128/JVI.02632-13
22. Henderson, J. O., Ball-Goodrich, L. J., & Parris, D. S. (1998). Structure-function analysis of the herpes simplex virus type 1 UL12 gene: correlation of deoxyribonuclease activity in vitro with replication function. Virology, 243(1), 247-259.
23. James, S. H., & Prichard, M. N. (2011). The genetic basis of human cytomegalovirus resistance and current trends in antiviral resistance analysis. Infectious Disorders Drug Targets, 11(5), 504-513.

24. Kelly, B. J., Fraefel, C., Cunningham, A. L., & Diefenbach, R. J. (2009). Functional roles of the tegument proteins of herpes simplex virus type 1. Virus Research, 145(2), 173-186. doi: 10.1016/j.virusres.2009.07.007

25. Kuchta, A. L., Parikh, H., Zhu, Y., Kellogg, G. E., Parris, D. S., & McVoy, M. A. (2012). Structural modelling and mutagenesis of human cytomegalovirus alkaline nuclease UL98. J Gen Virol, 93(Pt 1), 130-138. doi: 10.1099/vir.0.034876-0

26. Mettenleiter, T. C. K. B. G. G. H. (2009). Herpesvirus assembly: An update. VIRUS Virus Research, 143(2), 222-234.

27. Michaelis, M., Baumgarten, P., Mittelbronn, M., Driever, P. H., Doerr, H. W., & Cinatl, J., Jr. (2011). Oncomodulation by human cytomegalovirus: novel clinical findings open new roads. Med Microbiol Immunol, 200(1), 1-5. doi: 10.1007/s00430-010-0177-7

28. Muylaert, I., Tang, K.-W., & Elias, P. (2011). Replication and recombination of herpes simplex virus DNA. The Journal of biological chemistry, 286(18), 15619-15624. doi: 10.1074/jbc.R111.233981

29. Porter, I. M., & Stow, N. D. (2004). Replication, recombination and packaging of amplicon DNA in cells infected with the herpes simplex virus type 1 alkaline nuclease null mutant ambUL12. J Gen Virol, 85(Pt 12), 3501-3510.

30. Razonable, R. R. (2011). Antiviral drugs for viruses other than human immunodeficiency virus. Mayo Clinic Proceedings, 86(10), 1009-1026. doi: 10.4065/mcp.2011.0309

31. Sheaffer, A. K., Weinheimer, S. P., & Tenney, D. J. (1997). The human cytomegalovirus UL98 gene encodes the conserved herpesvirus alkaline nuclease. J Gen Virol, 78(Pt 1), 2953-2961.
32. Sijmons, S., Van Ranst, M., & Maes, P. (2014). Genomic and functional characteristics of human cytomegalovirus revealed by next-generation sequencing. Viruses, 6(3), 1049-1072. doi: 10.3390/v6031049
33. Sissons, J. G. P., Carmichael, A. J., McKinney, N., Sinclair, J. H., & Wills, M. R. (2002). Human cytomegalovirus and immunopathology. Springer Seminars In Immunopathology, 24(2), 169-185.
34. Steininger, C. (2007). Clinical relevance of cytomegalovirus infection in patients with disorders of the immune system. Clinical Microbiology And Infection: The Official Publication Of The European Society Of Clinical Microbiology And Infectious Diseases, 13(10), 953-963.
35. Sullivan, R. J., Pantanowitz, L., Casper, C., Stebbing, J., & Dezube, B. J. (2008). HIV/AIDS: epidemiology, pathophysiology, and treatment of Kaposi sarcoma-associated herpesvirus disease: Kaposi sarcoma, primary effusion lymphoma, and multicentric Castleman disease. Clin Infect Dis, 47(9), 1209-1215. doi: 10.1086/592298
36. Tolan, R. W. J. (2011). Cytomegalovirus Infection in the Fetus, Infant, Child, and Adolescent: An Overview of Virus Genetics and Pathogenesis, Disease Burden, Prevention, Diagnosis, Treatment, Antiviral Resistance, and Drug Targets. Infectious Disorders Drug Targets, 11(5), 424-425.

37. Van Damme, E., & Van Loock, M. (2014). Functional annotation of human cytomegalovirus gene products: an update. Frontiers In Microbiology, 5, 218-218. doi: 10.3389/fmicb.2014.00218

38. Wang, J. B., Zhu, Y., McVoy, M. A., & Parris, D. S. (2012). Changes in subcellular localization reveal interactions between human cytomegalovirus terminase subunits. Virol J, 9, 315-315. doi: 10.1186/1743-422X-9-315

39. Weinberg, J. M. (2007). Herpes zoster: Epidemiology, natural history, and common complications. Journal of the American Academy of Dermatology, 57(6), S130-S135. doi: 10.1016/j.jaad.2007.08.046

40. Weller, S. K., & Coen, D. M. (2012). Herpes simplex viruses: mechanisms of DNA replication. Cold Spring Harbor perspectives in biology, 4(9), a013011-a013011. doi: 10.1128/mBio.00278-11. (PMID: 22186611). Cites: MBio. 2012;3(1). pii: e00301-11. doi: 10.1128/mBio.00301-11. (PMID: 22318319). Subset: IM; Grant Information: AI069136 United States AI NIAID NIH HHS; AI19838 United States AI NIAID NIH HHS; AI21747 United States AI NIAID NIH HHS; AI26126 United States AI NIAID NIH HHS; R01 AI019838 United States AI NIAID NIH HHS; R01 AI021747 United States AI NIAID NIH HHS; R01 AI069136 United States AI NIAID NIH HHS Date of Electronic Publication: 2012 Sep 01. ; Original Imprints: Publication: Woodbury, NY : Cold Spring Harbor Laboratory Press 10.1101/cshperspect.a013011

41. Weller, S. K., & Kuchta, R. D. (2013). The DNA helicase-primase complex as a target for herpes viral infection. Expert Opinion On Therapeutic Targets, 17(10), 1119-1132. doi: 10.1517/14728222.2013.827663

42. Weller, S. K., Seghatoleslami, M. R., Shao, L., Rowse, D., & Carmichael, E. P. (1990). The herpes simplex virus type 1 alkaline nuclease is not essential for viral DNA synthesis: isolation and characterization of a lacZ insertion mutant. J Gen Virol, 71 (Pt 12), 2941-2952.
43. Williams, I. L. C. B. S. (2011). 6 Herpes viruses. HIV Medicine, 12, 61-69.
44. Xiong, H.-R., Luo, J., Hou, W., Xiao, H., & Yang, Z.-Q. (2011). The effect of emodin, an anthraquinone derivative extracted from the roots of Rheum tanguticum, against herpes simplex virus in vitro and in vivo. Journal Of Ethnopharmacology, 133(2), 718-723. doi: 10.1016/j.jep.2010.10.059
45. Yang, K., Wills, E. G., & Baines, J. D. (2011). A mutation in UL15 of herpes simplex virus 1 that reduces packaging of cleaved genomes. Journal Of Virology, 85(22), 11972-11980. doi: 10.1128/JVI.00857-11